

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



AD

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5: C07H 21/00, A61K 35/14 C07K 3/00, 7/00, 13/00 C12P 21/02, 21/06, C12N 15/00		A1	(11) International Publication Number: WO 93/08207 (43) International Publication Date: 29 April 1993 (29.04.93)
(21) International Application Number:	PCT/US92/08990		(74) Agent: PERKINS, Patricia, Anne; Immunex Corporation, 51 University Street, Seattle, WA 98101 (US).
(22) International Filing Date:	23 October 1992 (23.10.92)		(81) Designated States: AU, CA, FI, JP, KR, NO, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE).
(30) Priority data:	25 October 1991 (25.10.91) 5 December 1991 (05.12.91)	US US	Published <i>With international search report.</i>
(71) Applicant: IMMUNEX CORPORATION [US/US]; 51 University Street, Seattle, WA 98101 (US).			
(72) Inventors: ARMITAGE, Richard, J. ; 5133 Eagle Harbor Drive, Bainbridge Island, WA 98110 (US). FANSLOW, William, C. ; 32204, 22nd Avenue Southwest, Federal Way, WA 98023 (US). SPRIGGS, Melanie, K. ; 2256, 12th Avenue West, Seattle, WA 98119 (US).			

(54) Title: NOVEL CYTOKINE

(57) Abstract

There is disclosed a polypeptide (CD40-L) and DNA sequences, vectors and transformed host cells useful in providing CD40-L polypeptides. More particularly, this invention provides isolated human and murine CD40-L polypeptides that bind to the extracellular binding region of a CD40 receptor.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gahon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	LJ	Liechtenstein	SK	Slovak Republic
CI	Côte d'Ivoire	LK	Sri Lanka	SN	Senegal
CM	Cameroon	LU	Luxembourg	SU	Soviet Union
CS	Czechoslovakia	MC	Monaco	TD	Chad
CZ	Czech Republic	MG	Madagascar	TG	Togo
DE	Germany	ML	Mali	UA	Ukraine
DK	Denmark	MN	Mongolia	US	United States of America
ES	Spain			VN	Viet Nam
FI	Finland				

TITLE

## NOVEL CYTOKINE

5

TECHNICAL FIELD OF THE INVENTION

The present invention relates to a novel cytokine. More specifically, the present invention relates to the cloning of a murine and a human cytokine that binds to a human CD40 having both agonist and antagonist activity in soluble and membrane-bound forms.

10

BACKGROUND OF THE INVENTION

Cytokines that have an "Interleukin" designation are those protein factors that influence immune effector cells. Cytokines designated interleukin-1 through interleukin-12 have been reported and named as an interleukin. Other known cytokines include tumor necrosis factor (TNF), granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), mast cell growth factor (MGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), nerve growth factor (NGF), erythropoietin (EPO),  $\gamma$ -interferon ( $\gamma$ -IFN) and others.

15

20

DNAs for two different TNF receptors (Type I and Type II) have been cloned (Smith et al., *Science* 248:1019, 1990; and Schall et al., *Cell* 61:361, 1990). Both forms of TNF receptor are related to each other and belong to a family of receptors whose members include nerve growth factor receptor (Johnson et al., *Cell* 47:545, 1986), B cell antigen CD40 (Stamenkovic et al., *EMBO J.* 8:1403, 1989), T cell antigen OX40 (Mallett et al., *EMBO J.* 9:1063, 1990), human *Fas* antigen (Itoh et al., *Cell* 66:233, 1991) and murine 4-1BB receptor (Kwon et al., *Cell. Immunol.* 121:414, 1989 [Kwon et al. I] and Kwon et al., *Proc. Natl. Acad. Sci. USA* 86:1963, 1989 [Kwon et al. II]).

25

30

35

Human CD40 protein (CD40) is a peptide of 277 amino acids having a molecular weight of 30,600, and a 19 amino acid secretory signal peptide comprising predominantly hydrophobic amino acids (Stamenkovic et al.). The molecular weight (exclusive of glycosylation) of the mature human CD40 protein is 28,300. A cDNA encoding human CD40 was isolated from a cDNA library prepared from Burkitt lymphoma cell line Raji. The putative protein encoded by the CD40 cDNA contains a putative leader sequence, trans-membrane domain and a number of other features common to membrane-bound receptor proteins. CD40 has been found to be expressed on B lymphocytes, epithelial cells and some carcinoma cell lines.

A monoclonal antibody (mAb) directed against CD40 has been shown to mediate various functional effects of human B cells. These effects include: (a) homotypic adhesions (Gordon et al., *J. Immunol.* 140:1425, 1988 [Gordon et al. I]); (b) increased cell size (Gordon et al. I and Valle et al., *Eur. J. Immunol.* 19:1463, 1989); (c) proliferation of B cells activated with anti-IgM, anti-CD20 mAb, phorbol ester alone (Clark et al., *Proc. Natl. Acad. Sci. USA* 83:4494, 1986; and Paulie et al., *J. Immunol.* 142:590, 1989), or phorbol ester combined with interleukin-4 (Gordon et al., *Eur. J. Immunol.* 17:1535, 1987 [Gordon et al. II]; and (d) production of IgE (Jabara et al., *J. Exp. Med.* 172:1861, 1990; Zhang et al., *J. Immunol.* 146:1836, 1991) and IgM (Gascan et al., *J. Immunol.* 147:8, 1991) from interleukin-4 (IL-4) stimulated T-depleted cultures.

One such antibody, called mAb 89 by Banchereau et al., *Clin. Immunol. Spectrum* 3:8, 1991 [Banchereau et al. I], was found to induce human B cell proliferation at a relatively low antibody concentration (30 ng/ml or about  $10^{-10}$  M). 15 Proliferation lasted two to three weeks and resulted in a ten-fold expansion of the human B cell population. Optimal stimulation of the B cells occurred when CD40 surface molecule was cross-linked by IgM. Fab fragments of another anti-CD40 mAb induced only a weak proliferative response. Further, Banchereau et al., *Science* 251:70, 1991 [Banchereau et al. II] reported that resting human B cells entered a state 20 of sustained proliferation when incubated with both a murine fibroblastic Ltk<sup>-</sup> cell line that was transfected with human Fc receptor and with a monoclonal antibody specific for human CD40. Banchereau et al. II found that cross-linking CD40 is necessary for clonal expansion of B cells.

CD23 is a low affinity IgE receptor that has been found to be expressed on most 25 IgM<sup>-</sup>/IgD<sup>-</sup> mature B cells, but not T cells. CD23 has been sequenced and its sequence was described in Kikutani et al., *Cell* 47:657, 1986. Soluble CD23 (sCD23) was found to induce a pyrogenic reaction in rabbits and this reaction was abrogated by administration of human IgE (Ghaderi et al., *Immunology* 73:510, 1991). Therefore, CD23 may be an appropriate marker for soluble CD40 or CD40-L effects.

30 Prior to the present invention, a ligand for CD40 was unknown. Accordingly, there is a need in the art to identify and characterize a CD40 ligand (CD40-L).

#### SUMMARY OF THE INVENTION

A novel cytokine, hereafter referred to as "CD40-L," has been isolated and 35 characterized. The nucleotide sequence and deduced amino acid sequence of representative murine CD40-L cDNA is disclosed in SEQ ID NO:1 and Figure 1, and the amino acid sequence is also listed in SEQ ID NO:2. The nucleotide sequence and

deduced amino acid sequence of representative human CD40-L cDNA is disclosed in SEQ ID NO:11 and Figure 2, and the amino acid sequence is also listed in SEQ ID NO:12. The present invention further comprises other CD40-L polypeptides encoded by nucleotide sequences that hybridize, under moderate or severe stringency

5 conditions, to probes defined by SEQ ID NO:11 (the coding region of human CD40-L), fragments of the sequence extending from nucleotide 46 to nucleotide 828 of SEQ ID NO:11, or to DNA or RNA sequences complementary to Figure 2 (SEQ ID NO:11) or fragments thereof. The invention further comprises nucleic acid sequences which, due to the degeneracy of the genetic code, encode polypeptides substantially identical or 10 substantially similar to polypeptides encoded by the nucleic acid sequences described above, and sequences complementary to them.

CD40-L is a type II membrane polypeptide having an extracellular region at its C-terminus, a transmembrane region and an intracellular region at its N-terminus. A soluble version of murine CD40-L has been found in supernatants from EL-4 cells and 15 EL-4 cells sorted on the basis of a biotinylated CD40/Fc fusion protein described herein. Soluble CD40-L comprises an extracellular region of CD40-L or a fragment thereof. The protein sequence of murine CD40-L is described in Figure 1 and SEQ ID NO:2, and human CD40-L in Figure 2 and SEQ ID NO:12. The extracellular region of murine CD40-L extends from amino acid 47 to amino acid 260 in Figure 1 and SEQ ID 20 NO:2, and of human CD40-L from amino acid 47 to amino acid 261 in Figure 2 and SEQ ID NO:12. CD40-L biological activity is mediated by binding of this cytokine with CD40 and includes B cell proliferation and induction of antibody secretion, including IgE secretion.

The present invention further provides antisense or sense oligonucleotides 25 (deoxyribonucleotides or ribonucleotides) that correspond to a sequence of at least about 12 nucleotides selected from the nucleotide sequence of CD40-L or DNA or RNA sequences complementary to the nucleotide sequence of CD40-L as described in SEQ ID NO:1 and SEQ ID NO:11 and in Figures 1 and 2. Such antisense or sense oligonucleotides prevent transcription or translation of CD40-L mRNA or polypeptides.

Further still, the present invention provides CD40-L peptide fragments that correspond to a protein sequence of at least 10 amino acids selected from the amino acid sequence encoded by SEQ ID NO:1 or SEQ ID NO:11 that can act as immunogens to generate antibodies specific to the CD40-L immunogens. Such CD40-L immunogen fragments can serve as antigenic determinants in providing monoclonal antibodies 35 specific for CD40-L.

The invention also provides a human CD40/Fc fusion protein and a soluble CD40 protein (sCD40) comprising the extracellular region of human CD40. Both

sCD40 and CD40/Fc fusion protein can inhibit CD40-L or anti-CD40 mAb induced B cell stimulation, IL-4-induced IgE stimulation and IL-4 induced CD23 induction in B cells.

5

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates nucleotide and amino acid sequences corresponding to murine CD40-L. This protein is a type II polypeptide having its N-terminus as its intracellular domain, followed by a transmembrane region, and an extracellular domain at the C-terminus of the polypeptide. The extracellular domain, which is longer than either the intracellular domain or the transmembrane region, contains one potential N-linked glycosylation site and two potential disulfide bonds in view of four cysteine (Cys) residues.

Figure 2 illustrates nucleotide and amino acid sequences corresponding to human CD40-L. This protein is a type II polypeptide having its N-terminus as its intracellular domain, followed by a transmembrane region, and an extracellular domain at the C-terminus of the polypeptide. The extracellular domain, which is longer than either the intracellular domain or the transmembrane region, contains 1 potential N-linked glycosylation site and 2 potential disulfide bonds in view of 5 cysteine (Cys) residues.

Figure 3 illustrates a comparison of protein sequences of human and murine CD40-L showing 77.7% homology at the amino acid level.

Figure 4 illustrates proliferation of T cell depleted human peripheral blood mononuclear cells (PBMC) caused by incubation with CV1 cells transfected with full length murine CD40-L cDNA (SEQ ID NO:1) and expressing bound CD40-L (CD40-L<sup>+</sup>CV1 cells) when compared with CV1 cells transfected with empty vector (HAVEO) and not expressing bound murine CD40-L. The day 7 proliferation results show that CD40-L<sup>+</sup>CV1 cells significantly increase proliferation of T-cell depleted PBMC in the presence or absence of interleukin-4 (IL-4).

Figure 5 illustrates a second determination of T cell depleted PBMC proliferation with addition of bound murine CD40-L and 10 ng/ml of IL-4. These data show no co-mitogenic effect of IL-4 but continued strong mitogenic effect of bound CD40-L.

Figure 6 illustrates that bound CD40-L augments IgE secretion.

Figure 7 illustrates that membrane-bound CD40-L stimulates CD23 shedding in the presence of IL-4.

Figure 8 illustrates proliferation of murine splenic B cells caused by membrane-bound murine CD40-L or 7A1 cells, which is a helper T cell clone.

Figure 9 illustrates a comparison of murine EL40.9 cells, a sorted cell line that was sorted on the basis of expression of murine CD40-L and T cells 7A1 for induction of an antigen-specific response indicated by plaque forming cells (PFC) by anti-sheep red blood cells (SCBC).

5 Figure 10 illustrates a comparison of B cell proliferative activity of membrane-bound CD40-L and other cell types transfected with different cDNAs. Membrane-bound CD40-L showed significantly more B cell proliferative activity than a helper T cell clone or other control cells.

10 Figure 11 illustrates that 7C2 cells (a helper T cell clone) and CV1 cells transfected with murine CD40-L cDNA induce anti-SRBC plaque forming cells.

Figure 12 illustrates a comparison of two helper T cell clones with cells expressing membrane-bound CD40-L for inducing murine B cell proliferation.

15 Figure 13 illustrates induction of antigen-specific plaque forming cells by membrane-bound CD40-L and a helper T cell clone in the presence or absence of added interleukin-2 (IL-2).

Figure 14 shows effects of membrane-bound CD40-L stimulating B cell proliferation and IgE secretion. The effects of membrane-bound CD40-L were inhibited by CD40 receptor but not by TNF receptor.

20

#### DETAILED DESCRIPTION OF THE INVENTION

Novel polypeptides that can act as a ligand for murine and human CD40 have been isolated and sequenced. More particularly, cDNAs encoding these ligands have been cloned and sequenced. Further provided are methods for expression of recombinant CD40-L polypeptides. CD40-L polypeptide include other forms of mammalian CD40-L, such as derivatives or analogs of human or murine CD40-L. Murine and human CD40-L comprise a 214 and 215, respectively amino acid extracellular region at the C-terminus of full length, membrane-bound polypeptide. The extracellular region contains the domain that binds to CD40. Murine and human CD40-L further comprise a homologous hydrophobic 24 amino acid transmembrane region delineated by charged amino acids on either side and a 22 amino acid intracellular region at their N-termini. The present invention further comprises full length CD40-L polypeptides or fragments thereof comprising all or part of the extracellular region or derivatives of the extracellular region and mammalian cells transfected with a cDNA encoding murine or human CD40-L and expressing human or murine CD40-L as a membrane-bound protein.

30 The present invention comprises isolated DNA sequences encoding CD40-L polypeptides and DNA or RNA sequences complementary to such isolated DNA

6

sequences. The isolated DNA sequences and their complements are selected from the group consisting of (a) nucleotides 184 through 828, nucleotides 193 through 828 or nucleotides 193 through 762 of the DNA sequence set forth in Figure 2 (SEQ ID NO:11) and their complements, (b) DNA sequences which hybridize to the DNA sequences of (a) or their complements under conditions of moderate stringency and which encode a CD40-L polypeptide, analogs or derivatives thereof, and (c) DNA sequences which, due to the degeneracy of the genetic code, encode CD40-L polypeptides encoded by any of the foregoing DNA sequences and their complements. In addition, the present invention includes vectors comprising DNA sequences encoding CD40-L polypeptides and analogs, and host cells transfected with such vectors.

10 The novel cytokine disclosed herein is a ligand for CD40, a receptor that is a member of the TNF receptor super family. Therefore, CD40-L is likely to be responsible for transducing signal via CD40, which is known to be expressed, for example, by B lymphocytes. Full-length CD40-L is a membrane-bound polypeptide with an extracellular region at its C terminus, a transmembrane region, and an intracellular region at its N-terminus. A soluble version of CD40-L can be made from the extracellular region or a fragment thereof and a soluble CD40-L has been found in culture supernatants from cells that express a membrane-bound version of CD40-L.

15 20 The protein sequence of the extracellular region of murine CD40-L extends from amino acid 47 to amino acid 260 in Figure 1 and SEQ ID NO:2. The protein sequence of the extracellular region of human CD40-L extends from amino acid 47 to amino acid 261 in Figure 2 and SEQ ID NO:12. The biological activity of CD40-L is mediated by binding to CD40 or a species-specific homolog thereof and comprises proliferation of B cells and induction of immunoglobulin secretion from activated B cells. CD40-L (including soluble monomeric and oligomeric forms, as well as membrane-bound forms) can effect B cell proliferation and immunoglobulin secretion (except IgE secretion) without the presence of added IL-4, in contrast to anti-CD40 antibodies, which require IL-4 and cross-linking to mediate activity.

25 30 CD40-L refers to a genus of polypeptides which are capable of binding CD40, or mammalian homologs of CD40. As used herein, the term "CD40-L" includes soluble CD40-L polypeptides lacking transmembrane and intracellular regions, mammalian homologs of human CD40-L, analogs of human or murine CD40-L or derivatives of human or murine CD40-L.

35 CD40-L may also be obtained by mutations of nucleotide sequences coding for a CD40-L polypeptide. A CD40-L analog, as referred to herein, is a polypeptide substantially homologous to a sequence of human or murine CD40-L but which has an

amino acid sequence different from native sequence CD40-L (human or murine species) polypeptide because of one or a plurality of deletions, insertions or substitutions. Analogs of CD40-L can be synthesized from DNA constructs prepared by oligonucleotide synthesis and ligation or by site-specific mutagenesis techniques.

5 The primary amino acid structure of human or murine CD40-L may be modified to create CD40-L derivatives by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like, or by creating amino acid sequence mutants. Covalent derivatives of CD40-L are prepared by linking particular functional groups to CD40-L amino acid side chains or at 10 the N-terminus or C-terminus of a CD40-L polypeptide or the extracellular domain thereof. Other derivatives of CD40-L within the scope of this invention include covalent or aggregative conjugates of CD40-L or its fragments with other proteins or polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusions. For example, the conjugate may comprise a signal or leader polypeptide 15 sequence at the N-terminal region or C-terminal region of a CD40-L polypeptide which co-translationally or post-translationally directs transfer of the conjugate from its site of synthesis to a site inside or outside of the cell membrane or cell wall (e.g. the  $\alpha$ -factor leader of *Saccharomyces*). CD40-L polypeptide fusions can comprise polypeptides added to facilitate purification and identification of CD40-L (e.g. poly-His), or fusions 20 with other cytokines to provide novel polyfunctional entities. Other cytokines include, for example, any of interleukins-1 through 13, TNF (tumor necrosis factor), GM-CSF (granulocyte macrophage-colony stimulating factor), G-CSF (granulocyte-colony stimulating factor), MGF (mast cell growth factor), EGF (epidermal growth factor), PDGF (platelet-derived growth factor), NGF (nerve growth factor), EPO 25 (erythropoietin),  $\gamma$ -IFN (gamma interferon), 4-1BB-L (4-1BB ligand) and other cytokines that affect immune cell growth, differentiation or function.

Nucleic acid sequences within the scope of the present invention include DNA and/or RNA sequences that hybridize to the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:11 or the complementary strands, under conditions of moderate or severe 30 stringency. Moderate stringency hybridization conditions refer to conditions described in, for example, Sambrook et al. *Molecular Cloning: A Laboratory Manual*, 2 ed. Vol. 1, pp. 1.101-104, Cold Spring Harbor Laboratory Press, (1989). Conditions of moderate stringency, as defined by Sambrook et al., include use of a prewashing solution of 5 X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0) and hybridization 35 conditions of 50°C, 5 X SSC, overnight. Conditions of severe stringency include higher temperatures of hybridization and washing.

Biological activity of CD40-L may be determined, for example, by competition for binding to the ligand binding domain of CD40 (i.e. competitive binding assays). Both murine CD40-L and human CD40-L bind to human CD40. The binding affinity of murine CD40-L (expressed on sorted murine EL-40.9 cells) for human CD40 was approximately

5  $1.74 \times 10^9 \text{ M}^{-1}$ . Similarly, the binding affinity of murine CD40-L (expressed on unsorted murine EL-46.1 cells) for human CD40 was approximately  $2.3 \times 10^9 \text{ M}^{-1}$ . Both binding affinity measurements are within a range typical of cytokine/cytokine receptor binding.

10 One configuration of a competitive binding assay for CD40-L polypeptide uses a radiolabeled, soluble murine CD40-L according to Figure 1 (SEQ ID NO:1) or human CD40-L according to Figure 2 (SEQ ID NO:11), and intact cells expressing CD40 (e.g., human B cells). Instead of intact cells, one could substitute soluble CD40 (such as a CD40/Fc fusion protein) bound to a solid phase through a Protein A or Protein G interaction with the Fc region of the fusion protein. A second configuration of a competitive binding assay utilizes radiolabeled soluble CD40 such as a CD40/Fc fusion protein, and intact cells expressing CD40-L. Alternatively, soluble CD40-L could be bound to a solid phase.

15 Competitive binding assays can be performed using standard methodology. For example, radiolabeled murine CD40-L can be used to compete with a putative CD40-L homolog to assay for binding activity against surface-bound CD40. Qualitative results can be obtained by competitive autoradiographic plate binding assays, or Scatchard plots may be utilized to generate quantitative results.

20 Competitive binding assays with intact cells expressing CD40 can be performed by two methods. In the first method, B cells are grown either in suspension or by adherence to tissue culture plates. Adherent cells can be removed by treatment with 5 mM EDTA treatment for ten minutes at 37° C. In a second method, transfected COS cells expressing membrane-bound CD40 can be used. COS cells or another mammalian cell can be transfected with human CD40 cDNA in an appropriate vector to express full length CD40 with an extracellular region exterior to the cell.

25 Alternatively, soluble CD40 can be bound to a solid phase such as a column chromatography matrix, or a tube or similar substrate suitable for analysis for the presence of a detectable moiety such as  $^{125}\text{I}$ . Binding to a solid phase can be accomplished, for example, by obtaining a CD40/Fc fusion protein and binding it to a protein A or protein G surface.

30 Another means to measure the biological activity of CD40-L and homologs thereof is to utilize conjugated, soluble CD40 (for example,  $^{125}\text{I}$ -CD40/Fc) in

IP

6

7

8

9

competition assays similar to those described above. In this case, however, intact cells expressing CD40-L, or soluble CD40-L bound to a solid substrate, are used to measure competition for binding of conjugated, soluble CD40 to CD40-L by a sample containing a putative CD40 homolog.

CD40-L may also be assayed by measuring biological activity in a B cell proliferation assay. Human B cells may be obtained from human tonsils by purification by negative selection and Percoll density sedimentation, as described by Defrance et al., *J. Immunol.* 139:1135, 1987. Burkitt lymphoma cell lines may be used to measure cell proliferation in response to CD40-L. Examples of Burkitt lymphoma cell lines include, for example, Raji (ATCC CCL 86), Daudi (ATCC CCL 213) and Namalwa (ATCC CRL 1432). Membrane-bound CD40-L stimulated B cell proliferation. Oligomeric, preferably dimeric, CD40-L can stimulate B cell proliferation. CD40 (receptor) antagonizes CD40-L proliferation of B cells.

Yet another assay for determining CD40-L biological activity is to measure immunoglobulin produced by B cells in response to activation by CD40-L or a derivative or analog thereof. Polyclonal immunoglobulin secretion can be measured, for example, by incubating with  $5 \times 10^5$  B cells/ml in culture for at least seven days. Immunoglobulin (Ig) production can be measured by an ELISA assay such as one described in Maliszewski et al., *J. Immunol.* 144:3028, 1990 [Maliszewski et al. I] or Maliszewski et al., *Eur J. Immunol.* 20:1735, 1990 [Maliszewski et al. II]. Murine B cells can be obtained, for example, from mice and cultured according to procedures described in Grabstein et al., *J. Exp. Med.* 163:1405, 1986 [Grabstein et al. I], Maliszewski et al. I, and Maliszewski et al. II.

CD40-L can be used in a binding assay to detect cells expressing CD40. For example, murine CD40-L according to Figure 1 (SEQ ID NO:1) or human CD40-L according to Figure 2 (SEQ ID NO:11), or an extracellular domain or a fragment thereof, can be conjugated to a detectable moiety such as  $^{125}\text{I}$ . Radiolabeling with  $^{125}\text{I}$  can be performed by any of several standard methodologies that yield a functional  $^{125}\text{I}$ -CD40-L molecule labeled to high specific activity. Alternatively, another detectable moiety such as an enzyme that can catalyze a colorimetric or fluorometric reaction, biotin or avidin may be used. Cells expressing CD40 can be contacted with conjugated CD40-L. After incubation, unbound conjugated CD40-L is removed and binding is measured using the detectable moiety.

CD40-L polypeptides may exist as oligomers, such as dimers or trimers. Oligomers are linked by disulfide bonds formed between cysteine residues on different CD40-L polypeptides. Alternatively, one can link two soluble CD40-L domains with a Gly<sub>4</sub>SerGly<sub>5</sub>Ser linker sequence, or other linker sequence described in United States

Patent 5,073,627; which is incorporated by reference herein. CD40-L polypeptides may also be created by fusion of the C terminal of soluble CD40-L (extracellular domain) to the Fc region of IgG1 (for example, SEQ ID NO:3) as described for the CD40/Fc fusion protein. CD40-L/Fc fusion proteins are allowed to assemble much like heavy chains of an antibody molecule to form divalent CD40-L. If fusion proteins are made with both heavy and light chains of an antibody, it is possible to form a CD40-L oligomer with as many as four CD40-L extracellular regions.

Fusion proteins can be prepared using conventional techniques of enzyme cutting and ligation of fragments from desired sequences. PCR techniques employing synthetic oligonucleotides may be used to prepare and/or amplify the desired fragments. Overlapping synthetic oligonucleotides representing the desired sequences can also be used to prepare DNA constructs encoding fusion proteins. Fusion proteins can also comprise CD40-L and two or more additional sequences, including a leader (or signal peptide) sequence, Fc region, linker sequence, and sequences encoding highly antigenic moieties that provide a means for facile purification or rapid detection of a fusion protein.

Signal peptides facilitate secretion of proteins from cells. An exemplary signal peptide is the amino terminal 25 amino acids of the leader sequence of human interleukin-7 (IL-7; Goodwin et al., *Proc. Natl. Acad. Sci. U.S.A.* 86:302, 1989; Figure 2B). Other signal peptides may also be employed. For example, certain nucleotides in the IL-7 leader sequence can be altered without altering the amino acid sequence. Additionally, amino acid changes that do not affect the ability of the IL-7 sequence to act as a leader sequence can be made.

The Flag<sup>®</sup> octapeptide (Asp-Tyr-Lys-Asp-Asp-Asp-Lys) does not alter the biological activity of fusion proteins, is highly antigenic and provides an epitope reversibly bound by a specific monoclonal antibody, enabling rapid detection and facile purification of the expressed fusion protein. The Flag<sup>®</sup> sequence is also specifically cleaved by bovine mucosal enterokinase at the residue immediately following the Asp-Lys pairing, fusion proteins capped with this peptide may also be resistant to intracellular degradation in *E. coli*. A murine monoclonal antibody that binds the Flag<sup>®</sup> sequence has been deposited with the ATCC under accession number HB 9259; methods of using the antibody in purification of fusion proteins comprising the Flag<sup>®</sup> sequence are described in U.S. Patent 5,011,912, which is incorporated by reference herein.

Suitable Fc regions are defined as Fc regions that can bind to protein A or protein G, or alternatively, are recognized by an antibody that can be used in purification or detection of a fusion protein comprising the Fc region. Preferable Fc

regions include the Fc region of human IgG<sub>1</sub> or murine IgG<sub>1</sub>. One example is the human IgG<sub>1</sub> Fc region shown in SEQ ID NO:3; another example is an Fc region encoded by cDNA obtained by PCR from oligonucleotide primers from SEQ ID NO:9 and SEQ ID NO:10 with human cDNA as a template. Portions of a suitable Fc region 5 may also be used, for example, an Fc region of human IgG<sub>1</sub> from which has been deleted a sequence of amino acids responsible for binding to protein A, such that the resultant Fc region binds to protein G but not protein A.

The [Gly<sub>4</sub>Ser]<sub>3</sub> repeat sequence provides a linker sequence that separates the extracellular region of the CD40-L from the Fc portion of the fusion protein by a 10 distance sufficient to ensure that the CD40-L properly folds into its secondary and tertiary structures. Suitable linker sequences (1) will adopt a flexible extended conformation, (2) will not exhibit a propensity for developing an ordered secondary structure which could interact with the functional domains of fusion proteins, and (3) will have minimal hydrophobic or charged character which could promote interaction 15 with the functional protein domains. Typical surface amino acids in flexible protein regions include Gly, Asn and Ser. Virtually any permutation of amino acid sequences containing Gly, Asn and Ser would be expected to satisfy the above criteria for a linker sequence. Other near neutral amino acids, such as Thr and Ala, may also be used in the linker sequence. The length of the linker sequence may vary without significantly 20 affecting the biological activity of the fusion protein. Linker sequences are unnecessary where the proteins being fused have non-essential N- or C-terminal amino acid regions which can be used to separate the functional domains and prevent steric interference.

CD40-L polypeptides may exist as soluble polypeptides comprising the extracellular domain of CD40-L as shown in Figure 1 (SEQ ID NO:1) and Figure 2 25 (SEQ ID NO:11) or as membrane-bound polypeptides comprising the extracellular domain, a transmembrane region and a short intracellular domain, as shown in Figure 1 (SEQ ID NO:1) and Figure 2 (SEQ ID NO:11) for the murine and human sequences, respectively. Moreover, the present invention comprises oligomers of CD40-L extracellular domains or fragments thereof, linked by disulfide interactions, or 30 expressed as fusion polymers with or without spacer amino acid linking groups. For example, a dimer CD40-L molecule can be linked by an IgG Fc region linking group.

Without being bound by theory, membrane-bound CD40-L and oligomeric CD40-L can achieve activity stimulating Ig formation and proliferation of B cells previously only achieved by cross-linked anti-CD40 antibody in the presence of IL-4. 35 It further appears likely that monomeric soluble CD40-L, comprising only the extracellular domain of CD40-L and capable of binding to CD40 receptor, will serve to antagonize the activity of membrane-bound and oligomeric CD40-L and/or cross-linked

12

anti-CD40 antibodies. It further appears likely that the interaction of membrane-bound CD40-L with CD40 is the principal molecular interaction responsible for T cell contact dependent induction of B cell growth and differentiation to both antigen specific antibody production and polyclonal Ig secretion. In this regard, a mammalian cell 5 transfected with a cDNA encoding full length CD40-L (i.e., being membrane-bound and having an intracellular domain, a transmembrane region and an extracellular domain or a fragment thereof) can mimic T cells in their ability to induce B cell growth, differentiation and stimulation of antigen-specific antibody production. It appears that activities of oligomeric soluble CD40-L, preferably a dimer of extracellular regions, can 10 mimic the biological activities of membrane-bound CD40-L. Moreover, soluble monomeric CD40-L (comprising the extracellular domain or a fragment thereof) can bind to CD40 receptor to prevent T cell interaction with B cells and therefor have activity similar to CD40 (receptor) extracellular domain which itself may be in monomeric or in oligomeric form. Alternatively, CD40-L can be oligomeric (preferably 15 a dimer) to act as a soluble factor capable of inducing B cell growth, differentiation and stimulation of antigen-specific antibody production. Accordingly, it appears that membrane-bound CD40-L and oligomeric CD40-L act as CD40 agonists, while soluble (monomeric) CD40-L and soluble CD40 act as CD40 antagonists by blocking CD40 receptor sites without significantly transducing signal or by preventing CD40-L binding 20 to CD40 sites on B cells and other target cells.

Both CD40 agonists and CD40 antagonists will have useful therapeutic activity. For example, CD40 agonists (i.e., membrane-bound CD40-L and oligomeric CD40-L) are useful as vaccine adjuvants and for stimulating mAb production from hybridoma cells. CD40 antagonists (i.e., CD40 receptor, CD40/Fc and possibly soluble, 25 monomeric CD40-L) are useful for treating autoimmune diseases characterized by presence of high levels of antigen-antibody complexes, such as allergy, lupus, rheumatoid arthritis, insulin dependent diabetes mellitus (IDDM), graft versus host disease (GVHD) and others.

IgE secretion from human B cells can be induced by IL-4 in the presence of T 30 cells (Vercelli et al., *J. Exp. Med.* 169:1295, 1989). Further, IgE production can be induced from T cell depleted PBM (peripheral blood mononuclear cells) by addition of an anti-CD40 mAb (Jabara et al., *J. Exp. Med.* 172:1861, 1990 and Zhang et al., *J. Immunol.* 146:1836, 1991). The present invention further includes a method for inhibiting IgE production from activated B cells, activated by IL-4 in the presence of T 35 cells or by CD40-L (preferably, membrane-bound CD40-L), comprising administering an effective amount of a CD40/Fc fusion protein, as described herein, or a soluble CD40 encoded by the cDNA sequence described in SEQ ID NO. 3. Similarly, CD40

13

receptors and possibly soluble CD40-L (monomer only) can also block secretion of other antibody isotypes.

The present invention further includes CD40-L polypeptides with or without associated native-pattern glycosylation. CD40-L expressed in yeast or mammalian expression systems (e.g., COS-7 cells) may be similar to or significantly different from a native CD40-L polypeptide in molecular weight and glycosylation pattern, depending upon the choice of expression system. Expression of CD40-L polypeptides in bacterial expression systems, such as *E. coli*, provides non-glycosylated molecules.

DNA constructs that encode various additions or substitutions of amino acid residues or sequences, or deletions of terminal or internal residues or sequences not needed for biological activity or binding can be prepared. For example, the extracellular CD40-L N-glycosylation site can be modified to preclude glycosylation while allowing expression of a homogeneous, reduced carbohydrate analog using yeast expression systems. N-glycosylation sites in eukaryotic polypeptides are characterized by an amino acid triplet Asn-X-Y, wherein X is any amino acid except Pro and Y is Ser or Thr. Appropriate modifications to the nucleotide sequence encoding this triplet will result in substitutions, additions or deletions that prevent attachment of carbohydrate residues at the Asn side chain. In another example, sequences encoding Cys residues can be altered to cause the Cys residues to be deleted or replaced with other amino acids, preventing formation of incorrect intramolecular disulfide bridges upon renaturation. Human CD40-L comprises five Cys residues in its extracellular domain. Thus, at least one of the five Cys residues can be replaced with another amino acid or deleted without effecting protein tertiary structure or disulfide bond formation.

Other approaches to mutagenesis involve modification of sequences encoding dibasic amino acid residues to enhance expression in yeast systems in which KEX2 protease activity is present. Sub-units of a CD40-L polypeptide may be constructed by deleting sequences encoding terminal or internal residues or sequences.

CD40-L polypeptides are encoded by multi-exon genes. The present invention further includes alternative mRNA constructs which can be attributed to different mRNA splicing events following transcription and which share regions of identity or similarity with the cDNAs disclosed herein.

Antisense or sense oligonucleotides comprise a single-stranded nucleic acid sequence (either RNA or DNA) capable of binding to target CD40-L mRNA (sense) or CD40-L DNA (antisense) sequences. Antisense or sense oligonucleotides, according to the present invention, comprise a fragment of SEQ ID NO:1 or SEQ ID NO:11, or a DNA or RNA complement of SEQ ID NO:1 or SEQ ID NO:11. Such a fragment comprises at least about 14 nucleotides. Preferably, such a fragment comprises from

about 14 to about 30 nucleotides. The ability to create an antisense or a sense oligonucleotide, based upon a cDNA sequence for CD40-L is described in, for example, Stein and Cohen, *Cancer Res.* 48:2659, 1988 and van der Krol et al., *BioTechniques* 6:958, 1988.

5 Binding of antisense or sense oligonucleotides to target nucleic acid sequences results in the formation of duplexes that block translation (RNA) or transcription (DNA) by one of several means, including enhanced degradation of the duplexes, premature termination of transcription or translation, or by other means. Suitable polymerase promotors include promotors for any RNA polymerase, or promotors for  
10 any DNA polymerase. Antisense or sense oligonucleotides further comprise oligonucleotides having modified sugar-phosphodiester backbones (or other sugar linkages, such as those described in WO91/06629) and wherein such sugar linkages are resistant to endogenous nucleases. Such oligonucleotides with resistant sugar linkages are stable *in vivo* (i.e., capable of resisting enzymatic degradation) but retain sequence  
15 specificity to be able to bind to target nucleotide sequences. Other examples of sense or antisense oligonucleotides include those oligonucleotides which are covalently linked to organic moieties, such as those described in WO 90/10448, and other moieties that increases affinity of the oligonucleotide for a target nucleic acid sequence, such as poly-(L-lysine). Further still, intercalating agents, such as ellipticine, and alkylating agents  
20 or metal complexes may be attached to sense or antisense oligonucleotides to modify binding specificities of the antisense or sense oligonucleotide for the target nucleotide sequence. Antisense or sense oligonucleotides may be introduced into a cell containing the target nucleic acid sequence by any gene transfer method, including, for example, CaPO<sub>4</sub>-mediated DNA transfection, electroporation, or other gene transfer vectors such  
25 as Epstein-Barr virus. Antisense or sense oligonucleotides are preferably introduced into a cell containing the target nucleic acid sequence by insertion of the antisense or sense oligonucleotide into a suitable retroviral vector, then contacting the cell with the retrovirus vector containing the inserted sequence, either *in vivo* or *ex vivo*. Suitable retroviral vectors include, but are not limited to, the murine retrovirus M-MuLV, N2 (a  
30 retrovirus derived from M-MuLV), or the double copy vectors designated DCTS<sub>A</sub>, DCTS<sub>B</sub> and DCTS<sub>C</sub> (see PCT Application US 90/02656). Alternatively, other promoter sequences may be used to express the oligonucleotide.

Sense or antisense oligonucleotides may also be introduced into a cell containing the target nucleotide sequence by formation of a conjugate with a ligand binding molecule, as described in WO 91/04753. Suitable ligand binding molecules include, but are not limited to, cell surface receptors, growth factors, other cytokines, or other ligands that bind to cell surface receptors. Preferably, conjugation of the

ip

□

□

6

3

ligand binding molecule does not substantially interfere with the ability of the ligand binding molecule to bind to its corresponding molecule or receptor, or block entry of the sense or antisense oligonucleotide or its conjugated version into the cell.

Alternatively, a sense or an antisense oligonucleotide may be introduced into a 5 cell containing the target nucleic acid sequence by formation of an oligonucleotide-lipid complex, as described in WO 90/10448. The sense or antisense oligonucleotide-lipid complex is preferably dissociated within the cell by an endogenous lipase.

The sequence of murine CD40-L cDNA was obtained by direct expression techniques. The sequence of human CD40-L was obtained by cross-species 10 hybridization techniques using the murine CD40-L cDNA as a probe.

We cloned murine CD40-L by first obtaining a clone of the extracellular region of human CD40 (the receptor) by polymerase chain reaction (PCR) techniques using primers based upon a sequence published in Stamenkovic et al. (SEQ ID NO:4). An upstream oligonucleotide primer 5'-CCGTCGACCAACCATGGTTCGTCTGCC -3' 15 (SEQ ID NO:5) introduces a *Sal* 1 site upstream from an initiator methionine of CD40 and a downstream oligonucleotide primer 5'-CCGTCGACGTCTAGAGCCGATCCTGGGG-3' (SEQ ID NO:6) inserts a termination codon after amino acid 192 of CD40, followed by *Xba* 1 and *Sal* 1 sites. The amplified cDNA was digested with *Sal* 1 and cloned into pDC406 (McMahan et 20 al., *EMBO J.* 10:2821, 1991) to construct pDC406/s CD40.

A second CD40 receptor fragment (SEQ ID NO:4) was obtained by PCR techniques for fusion to the Fc domain of human IgG1 (SEQ ID NO:3). Briefly, The upstream oligonucleotide primer (SEQ ID NO:5) and fusion template (SEQ ID NO:4) were the same as before. The downstream oligonucleotide primer was 5'- 25 ACAAGATCTGGGCTCTACGTATCTCAGCCGATCCTGGGAC-3' (SEQ ID NO:7) that inserts amino acids Tyr Val Glu Pro Arg (SEQ ID NO:8) after amino acid 193 of CD40. Glu and Pro are the first two amino acids of a hinge region of human IgG1, and are followed by a *Bgl* II restriction site. The *Bgl* II restriction site was used to fuse the extracellular domain of CD40 to the remainder of human IgG1 Fc region.

Other fusion proteins comprising ligand binding domains from other receptors 30 can be made by obtaining a DNA sequence for the ligand binding domain of a receptor and fusing this sequence to a DNA sequence encoding an Fc region of an antibody molecule that binds to protein A or protein G, or another polypeptide that is capable of affinity purification, for example, avidin or streptavidin. The resultant gene construct 35 can be introduced into mammalian cells to transiently express a fusion protein. Receptor/Fc fusion proteins can be purified by protein A or protein G affinity purification. Receptor/avidin fusion proteins can be purified by biotin affinity

chromatography. The fusion protein can later be removed from the column by eluting with a high salt solution or another appropriate buffer.

We obtained a cDNA encoding human IgG1 Fc region by PCR amplification using cDNA from human cells as a template and an upstream oligonucleotide primer 5'-

5 TATTAATCATTCACTAGGGCCCAGATCTGTGACAAA~~A~~CTCAC-3' (SEQ ID NO:9) and a downstream oligonucleotide primer 5'-

GCCAGCTTAACTAGTTCATTACCCGGAGACAGGGAGA-3" (SEQ ID NO:10).

The PCR amplified cDNA introduced a *Bgl* II site near the beginning of the hinge region, which was used to ligate CD40 extracellular domain to construct a s CD40/Fc

10 fusion cDNA, which was ligated into pDC406 to construct pDC406/CD40/Fc. Other suitable Fc regions are defined as any region that can bind with high affinity to protein A or protein G, and includes the Fc region of human IgG1 or murine IgG1. One example is the human IgG1 Fc region shown in SEQ ID NO:3 or the cDNA obtained by PCR from oligonucleotide primers from SEQ ID NO:9 and SEQ ID NO:10 with

15 human cDNA as a template.

Receptor/Fc fusion molecules preferably are synthesized in recombinant mammalian cell culture because they are generally too large and complex to be synthesized by prokaryotic expression methods. Examples of suitable mammalian cells for expressing a receptor/Fc fusion protein include CV-1 cells (ATCC CCL 70) and COS-7 cells (ATCC CRL 1651), both derived from monkey kidney.

20 The DNA construct pDC406/CD40/Fc was transfected into the monkey kidney cell line CV-1/EBNA (ATCC CRL 10478). The pDC406 plasmid includes regulatory sequences derived from SV40, human immunodeficiency virus (HIV), and Epstein-Barr virus (EBV). The CV-1/EBNA cell line was derived by transfection of the CV-1 cell line with a gene encoding Epstein-Barr virus nuclear antigen-1 (EBNA-1) and 25 constitutively express EBNA-1 driven from human CMV immediate-early enhancer/promoter. An EBNA-1 gene allows for episomal replication of expression vectors, such as pDC406, that contain the EBV origin of replication.

Transfectants expressing CD40/Fc fusion protein are initially identified using 30 dot blots or Western blots. The supernatants are then subjected to dot blot or gel electrophoresis followed by transfer of the electrophoresed proteins for binding to G28-5 mAb (an antibody that binds to human CD40 receptor). The blotted proteins were then incubated with radiolabeled with  $^{125}\text{I}$ -protein A, washed to remove unbound label, and examined for expression of Fc. Monoclonal antibody G28-5 was produced 35 according to Clark et al., *supra*.

Once cells expressing the fusion construct were identified, large scale cultures of transfected cells were grown to accumulate supernatant from cells expressing

CD40/Fc. CD40/Fc fusion protein in supernatant fluid was purified by affinity purification. Briefly, one liter of culture supernatant containing CD40/Fc fusion protein was purified by filtering mammalian cell supernatants (e.g., in a 0.45 $\mu$  filter) and applying filtrate to a protein A/G antibody affinity column (Schleicher and Schuell, Keene, NH) at 4°C at a flow rate of 80 ml/hr for a 1.5 cm x 12.0 cm column. The column was washed with 0.5 M NaCl in PBS until free protein could not be detected in wash buffer. Finally, the column was washed with PBS. Bound fusion protein was eluted from the column with 25 mM citrate buffer, pH 2.8, and brought to pH 7 with 500 mM Hepes buffer, pH 9.1. Silver-stained SDS gels of the eluted CD40/Fc fusion protein showed it to be > 98% pure.

Soluble CD40 (sCD40) and CD40/Fc fusion proteins were made as described herein. The supernatants were purified through a G28-5 (anti-CD40 mAb) affinity column to affinity purify sCD40 expressed by the transfected CV-1/EBNA cells. Protein-containing fractions were pooled and aliquots removed for G28-5 binding assays and analysis by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) in the presence of 1 mM dithiothreitol as a reducing agent. A single band was seen of molecular weight 28,100 daltons. In the absence of a reducing agent, SDS-PAGE analysis of sCD40 revealed two bands, a major band of molecular weight 56,000 and a minor band of molecular weight 28,000. The banding pattern indicates that the majority of sCD40 exists as a disulfide-linked homodimer in solution. The 28,000 band is free monomer.

CD40 proteins were visualized by silver staining. Sample protein concentrations were determined using a micro-BCA assay (Pierce) with ultrapure bovine serum albumin as standard. Soluble CD40 purity and protein concentration were confirmed by amino acid analysis. Purified soluble CD40 was absorbed to PVDF paper and the paper subjected to automated Edman degradation on an Applied Biosystems model 477A protein sequencer according to manufacturers instructions for N-terminal protein sequencing. This procedure checked the protein sequence of sCD40.

Soluble CD40 and CD40/Fc fusion protein were able to modulate human B cell responses in the absence of anti-CD40 mAb (G28-5). Purified tonsillar B cells were cultured with anti-IgM and human IL-4 and either sCD40 or CD40/Fc fusion protein was added. Neither form of CD40 had an inhibitory effect on B cell proliferation (as measured by tritiated thymidine incorporation). IL-4 receptor, by contrast, inhibited IL-4-induced B cell proliferation in a concentration-dependent manner.

Soluble CD40 and CD40/Fc were tested for their ability to inhibit IL-4 induced IgE secretion in a 2-donor MLC (mixed lymphocyte culture) system. In three

experiments, the level of IgE production was reduced as the concentration of CD40 was increased. Soluble CD40, added at a concentration of 10  $\mu$ g/ml, was able to completely inhibit IgE secretion in this model of allergy. Further, CD40/Fc had similar effects as its soluble counterpart. However, addition of an IL-7 receptor-Fc fusion protein (made by similar procedures with a published IL-7 receptor sequence) did not affect secretion of IgE in this model.

Levels of CD23 were also measured in the same MLC in response to sCD40 or CD40/Fc fusion proteins. Soluble CD40 produced a small, but reproducible decrease in sCD23 level at day 6 compared to cultures stimulated with IL-4 alone, however a stronger inhibitory effect was pronounced at day 12 in the same cultures. Soluble CD23 induction by IL-4-stimulated T-depleted PBM (peripheral blood macrophages) E-cells was similarly affected by addition of sCD40, causing a small decrease in sCD23 levels at day 6 and a more pronounced inhibition at day 12. In each culture system, the results with CD40/Fc fusion protein were substantially the same as with sCD40.

In an effort to isolate a cDNA for a CD40-L, purified CD40/Fc fusion protein was radioiodinated with  $^{125}$ I using a commercially available solid phase agent (IODO-GEN, Pierce). In this procedure, 5  $\mu$ g of IODO-GEN were plated at the bottom of a 10 x 75 mm glass tube and incubated for twenty minutes at 4° C with 75  $\mu$ l of 0.1 M sodium phosphate, pH 7.4 and 20  $\mu$ l (2 mCi)  $Na^{125}I$ . The solution was then transferred to a second glass tube containing 5  $\mu$ g of CD40/Fc in 45  $\mu$ l PBS (phosphate buffered saline) and this reaction mixture was incubated for twenty minutes at 4° C. The reaction mixture was fractionated by gel filtration on a 2 ml bed volume of Sephadex® G-25 (Sigma), and then equilibrated in RPMI 1640 medium containing 2.5% (v/v) bovine serum albumin (BSA), 0.2% (v/v) sodium azide and 20 mM Hepes, pH 7.4 binding medium. The final pool of  $^{125}$ I CD40/Fc was diluted to a working stock solution of  $1 \times 10^{-7}$  M in binding medium and stored for up to one month at 4° C without detectable loss of receptor binding activity.

A cDNA library was prepared from a EL4 cell line sorted by FACS (fluorescence activated cell sorting) on the basis of binding of a biotinylated CD40/Fc fusion protein. Cells were sorted five times until there was a significant shift in fluorescence intensity based upon expression of a ligand for CD40 by the sorted EL-4 cells. The five-times sorted cells were called EL-40.5 cells and these cells were cultured for the purposes of creating a cDNA library from EL-40.5 mRNA. Briefly, cDNA was synthesized, inserted into empty pDC406 vector and transformed into *E. coli*. Transformants were pooled, and the DNA from the pools was isolated and transfected into CV1-EBNA cells to create an expression cloning library. Transfected CV1-EBNA cells were cultured on slides for three days to permit transient expression

of CD40-L. The slides containing the transfected cells were then incubated with radioiodinated CD40/Fc, washed to remove unbound CD40/Fc, and fixed with gluteraldehyde. The fixed slides were dipped in liquid photographic emulsion and exposed in the dark. After developing the slides, they were individually examined with a microscope and cells expressing CD40-L were identified by the presence of autoradiographic silver grains against a light background.

5 The expression cloning library from EL-40.5 cells was screened and one pool, containing approximately 2000 individual clones, was identified as positive for binding 10  $^{125}$ I labeled CD40/Fc fusion protein. This pool was broken down into smaller pools of approximately 200 colonies. The smaller pools were screened as described above. One of the smaller pools was positive for CD40-L.

A single clone was isolated and sequenced by standard techniques, to provide the cDNA sequence and deduced amino acid sequence of murine CD40-L as shown in Figure 1 and SEQ ID NO:1.

15 The human homolog CD40-L cDNA was found by cross species hybridization techniques. Briefly, a human peripheral blood lymphocyte (PBL) cDNA library was made from peripheral blood lymphocytes treated with OKT3 antibody (ATCC, Rockville MD) that binds to CD3 (10 ng/ml) and interleukin-2 (IL-2, 10 ng/ml) for six days. The PBL cells were washed and then stimulated for 4 hours with 10 ng/ml PMA (phorbol myristate acetate, Sigma St Louis) and 500 ng/ml ionomycin (Calbiochem).  
20 Messenger RNA was isolated from stimulated PBL cells, cDNA formed and cDNA was ligated into Eco R1 linkers. Ligated cDNA was inserted into the Eco R1 site of  $\lambda$ gt10 phage cloning vehicle (Gigapak<sup>®</sup> Stratagene, San Diego, CA) according to manufacturer's instructions. Phage were amplified, plated at densities of approximately 20,000 phage per 15 cm plate, and phage lifts were performed, as  
25 described in Maniatis et al., *Molecular Biology: A Laboratory Manual*, Cold Spring Harbor Laboratory, NY, 1982, pages 316-328. A murine probe was constructed corresponding to the coding region of murine CD40-L from nucleotide 13 to nucleotide 793 of SEQ ID NO:1 and Figure 1. This probe was hybridized to the PBL library  
30 phage lifts under conditions of moderate to severe stringency. Briefly, hybridization conditions were 6 X SSC, 1 X Denhardt's solution, 2 mM EDTA, 0.5% NP40 (Nonidet P-40 detergent) at 63 °C overnight. This was followed by washing in 3 X SSC, 0.1% SDS for three hours at 55 °C followed by overnight exposure to X-Ray film. Positive plaques were identified at a frequency of approximately 1 per 1000  
35 plaques. Positive plaques were purified twice and cDNA was prepared from amplified cultures.

20

One can utilize the murine or human CD40-L cDNA sequences disclosed herein to obtain cDNAs encoding other mammalian homologs of murine or human CD40-L by cross-species hybridization techniques. Briefly, an oligonucleotide probe is created from the nucleotide sequence of the extracellular region of murine CD40-L as described in Figure 1 (SEQ ID NO:1) or human CD40-L as described in Figure 2 (SEQ ID NO:11). This probe can be made by standard techniques, such as those described in Maniatis et al. *supra*. The murine or human probe is used to screen a mammalian cDNA library or genomic library under moderate stringency conditions. Examples of mammalian cDNA or genomic libraries include, for cDNA, a library made from the mammal's peripheral blood lymphocytes. Alternatively, various cDNA libraries or mRNAs isolated from various cell lines can be screened by Northern hybridization to determine a suitable source of mammalian CD40-L DNA or mRNA.

Recombinant expression vectors for expression of CD40-L by recombinant DNA techniques include a CD40-L DNA sequence comprising a synthetic or cDNA-derived DNA fragment encoding a CD40-L polypeptide, operably linked to a suitable transcriptional or translational regulatory nucleotide sequence, such as one derived from a mammalian, microbial, viral, or insect gene. Examples of regulatory sequences include sequences having a regulatory role in gene expression (e.g., a transcriptional promoter or enhancer), optionally an operator sequence to control transcription, a sequence encoding an mRNA ribosomal binding site, and appropriate sequences which control transcription and translation initiation and termination. Nucleotide sequences are operably linked when the regulatory sequence functionally relates to the CD40-L DNA sequence. Thus, a promoter nucleotide sequence is operably linked to a CD40-L DNA sequence if the promoter nucleotide sequence controls the transcription of the CD40-L DNA sequence. Still further, a ribosome binding site may be operably linked to a sequence for a CD40-L polypeptide if the ribosome binding site is positioned within the vector to encourage translation. In addition, sequences encoding signal peptides can be incorporated into expression vectors. For example, a DNA sequence for a signal peptide (secretory leader) may be operably linked to a CD40-L DNA sequence. The signal peptide is expressed as a precursor amino acid sequence which enables improved extracellular secretion of translated fusion polypeptide by a yeast host cell.

Suitable host cells for expression of CD40-L polypeptides include prokaryotes, yeast or higher eukaryotic cells. Prokaryotes include gram negative or gram positive organisms, for example, *E. coli* or *Bacilli*. Suitable prokaryotic host cells for transformation include, for example, *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium*, and various other species within the genera *Pseudomonas*,

5 *Streptomyces*, and *Staphylococcus*. Higher eukaryotic cells include established cell lines of mammalian origin. Cell-free translation systems could also be employed to produce CD40-L polypeptides using RNAs derived from DNA constructs disclosed herein. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described, for example, in Pouwels et al.

Cloning Vectors: A Laboratory Manual, Elsevier, New York, (1985).

10 In a prokaryotic host cell, such as *E. coli*, a CD40-L polypeptide or analog may include an N-terminal methionine residue to facilitate expression of the recombinant polypeptide in the prokaryotic host cell. The N-terminal Met may be cleaved from the 15 expressed recombinant CD40-L polypeptide. Prokaryotic host cells may be used for expression of CD40-L polypeptides that do not require extensive proteolytic or disulfide processing.

15 The expression vectors carrying the recombinant CD40-L DNA sequence are transfected or transformed into a substantially homogeneous culture of a suitable host microorganism or mammalian cell line. Transformed host cells are cells which have been transformed or transfected with nucleotide sequences encoding CD40-L 20 polypeptides and express CD40-L polypeptides. Expressed CD40-L polypeptides will be located within the host cell and/or secreted into culture supernatant fluid, depending upon the nature of the host cell and the gene construct inserted into the host cell.

25 Expression vectors transfected into prokaryotic host cells generally comprise one or more phenotypic selectable markers. A phenotypic selectable marker is, for example, a gene encoding a protein that confers antibiotic resistance or that supplies an autotrophic requirement, and an origin of replication recognized by the host to ensure amplification within the host. Other useful expression vectors for prokaryotic host cells 30 include a selectable marker of bacterial origin derived from commercially available plasmids. This selectable marker can comprise genetic elements of the cloning vector pBR322 (ATCC 37017). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides simple means for identifying transformed cells. The pBR322 "backbone" sections are combined with an appropriate promoter and a CD40-L DNA sequence. Other commercially vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega Biotec, Madison, WI, USA).

35 Promoter sequences are commonly used for recombinant prokaryotic host cell expression vectors. Common promoter sequences include  $\beta$ -lactamase (penicillinase), lactose promoter system (Chang et al., *Nature* 275:615, 1978; and Goeddel et al., *Nature* 281:544, 1979), tryptophan (*trp*) promoter system (Goeddel et al., *Nucl. Acids Res.* 8:4057, 1980; and EP-A-36776) and tac promoter (Maniatis, *Molecular Cloning*:

22

5 *A Laboratory Manual*, Cold Spring Harbor Laboratory, p. 412, 1982). A particularly useful prokaryotic host cell expression system employs a phage  $\lambda$   $P_L$  promoter and a cI857ts thermolabile repressor sequence. Plasmid vectors available from the American Type Culture Collection which incorporate derivatives of the  $\lambda$   $P_L$  promoter include  
in *E. coli* RR1 (ATCC 53082)).

10 CD40-L may be expressed in yeast host cells, preferably from the *Saccharomyces* genus (e.g., *S. cerevisiae*). Other genera of yeast, such as *Pichia* or *Kluyveromyces*, may also be employed. Yeast vectors will often contain an origin of replication sequence from a  $2\mu$  yeast plasmid, an autonomously replicating sequence (ARS), a promoter region, sequences for polyadenylation, and sequences for transcription termination. Preferably, yeast vectors include an origin of replication sequence and selectable marker. Suitable promoter sequences for yeast vectors include promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem.* 255:2073, 1980) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg.* 7:149, 1968; and Holland et al., *Biochem.* 17:4900, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and 20 glucokinase. Other suitable vectors and promoters for use in yeast expression are further described in Hitzeman, EPA-73,657.

25 Yeast vectors can be assembled, for example, using DNA sequences from pBR322 for selection and replication in *E. coli* (Amp<sup>r</sup> gene and origin of replication). Other yeast DNA sequences that can be included in a yeast expression construct include a glucose-repressible ADH2 promoter and  $\alpha$ -factor secretion leader. The ADH2 promoter has been described by Russell et al. (*J. Biol. Chem.* 258:2674, 1982) and Beier et al. (*Nature* 300:724, 1982). The yeast  $\alpha$ -factor leader sequence directs secretion of heterologous polypeptides. The  $\alpha$ -factor leader sequence is often inserted between the promoter sequence and the structural gene sequence. See, e.g., Kurjan et al., *Cell* 30:933, 1982 and Bitter et al., *Proc. Natl. Acad. Sci. USA* 81:5330, 1984. Other leader sequences suitable for facilitating secretion of recombinant polypeptides from yeast hosts are known to those of skill in the art. A leader sequence may be modified near its 3' end to contain one or more restriction sites. This will facilitate fusion of the leader sequence to the structural gene.

30 35 Yeast transformation protocols are known to those of skill in the art. One such protocol is described by Hinnen et al., *Proc. Natl. Acad. Sci. USA* 75:1929, 1978. The Hinnen et al. protocol selects for Trp<sup>+</sup> transformants in a selective medium.

23

wherein the selective medium consists of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10  $\mu$ g/ml adenine and 20  $\mu$ g/ml uracil.

Yeast host cells transformed by vectors containing ADH2 promoter sequence may be grown for inducing expression in a "rich" medium. An example of a rich 5 medium is one consisting of 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80  $\mu$ g/ml adenine and 80  $\mu$ g/ml uracil. Derepression of the ADH2 promoter occurs when glucose is exhausted from the medium.

Mammalian or insect host cell culture systems could also be employed to express recombinant CD40-L polypeptides. Examples of suitable mammalian host cell 10 lines include the COS-7 line of monkey kidney cells (ATCC CRL 1651) (Gluzman et al., *Cell* 23:175, 1981), L cells, C127 cells, 3T3 cells (ATCC CCL 163), Chinese hamster ovary (CHO) cells, HeLa cells, and BHK (ATCC CRL 10) cell lines. Suitable 15 mammalian expression vectors include nontranscribed elements such as an origin of replication, a promoter sequence, an enhancer linked to the structural gene, other 5' or 3' flanking nontranscribed sequences, such as ribosome binding sites, a polyadenylation site, splice donor and acceptor sites, and transcriptional termination sequences.

Transcriptional and translational control sequences for mammalian host cell 20 expression vectors may be excised from viral genomes. For example, commonly used mammalian cell promoter sequences and enhancer sequences are derived from Polyoma virus, Adenovirus 2, Simian Virus 40 (SV40), and human cytomegalovirus. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites may be used to provide the other genetic elements required for expression of a structural gene sequence in a 25 mammalian host cell. Viral early and late promoters are particularly useful because both are easily obtained from a viral genome as a fragment which may also contain a viral origin of replication (Fiers et al., *Nature* 273:113, 1978). Smaller or larger SV40 fragments may also be used, provided the approximately 250 bp sequence extending from the *Hind* III site toward the *Bgl* I site located in the SV40 viral origin of 30 replication site is included.

Exemplary mammalian expression vectors can be constructed as disclosed by Okayama and Berg (*Mol. Cell. Biol.* 3:280, 1983). A useful high expression vector, PMLSV N1/N4, described by Cosman et al., *Nature* 312:768, 1984 has been deposited as ATCC 39890. Additional useful mammalian expression vectors are described in EP-35 A-0367566, and in U.S. Patent Application Serial No. 07/701,415, filed May 16, 1991, incorporated by reference herein. For expression of a type II protein extracellular region, such as CD40-L, a heterologous signal sequence should be added.

such as the signal sequence for interleukin-7 (IL-7) described in United States Patent 4,965,195, or the signal sequence for interleukin-2 receptor described in United States Patent Application 06/626,667 filed on July 2, 1984.

Human or murine CD40-L can be made in membrane-bound form when an 5 intracellular and transmembrane regions are included or in soluble form with only the extracellular domain. We expressed full length murine CD40-L in mammalian cells to yield cells expressing membrane-bound murine CD40-L. CV1 cells were transfected with a cDNA shown in Figure 1 (SEQ ID NO:1) in HAVEO vector or CV1 cells were transfected with HAVEO empty vector using techniques described in Example 6 herein. 10 This yielded transfected CV1 cells expressing membrane-bound murine CD40-L. These cells were used as a source of membrane-bound murine CD40-L for the series of experiments reported in Examples 10 -13 reported below.

Purification of Recombinant CD40-L Polypeptides

CD40-L polypeptides may be prepared by culturing transformed host cells 15 under culture conditions necessary to express CD40-L polypeptides. The resulting expressed polypeptides may then be purified from culture media or cell extracts. A CD40-L polypeptide, if desired, may be concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a purification 20 matrix such as a gel filtration medium. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices 25 comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred.

Finally, one or more reverse-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, (e.g., silica gel having pendant methyl or other aliphatic groups) can be employed to further purify CD40-L. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a substantially homogeneous recombinant protein.

It is also possible to utilize an affinity column comprising CD40 ligand binding domain to affinity-purify expressed CD40-L polypeptides. CD40-L polypeptides can be removed from an affinity column in a high salt elution buffer and then dialyzed into a lower salt buffer for use.

35 Recombinant protein produced in bacterial culture is usually isolated by initial disruption of the host cells, centrifugation, extraction from cell pellets if an insoluble polypeptide, or from the supernatant fluid if a soluble polypeptide, followed by one or

more concentration, salting-out, ion exchange, affinity purification or size exclusion chromatography steps. Finally, RP-HPLC can be employed for final purification steps. Microbial cells can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

5        Transformed yeast host cells are preferably employed to express CD40-L as a secreted polypeptide. This simplifies purification. Secreted recombinant polypeptide from a yeast host cell fermentation can be purified by methods analogous to those disclosed by Urdal et al. (*J. Chromatog.* 296:171, 1984). Urdal et al. describe two sequential, reversed-phase HPLC steps for purification of recombinant human IL-2 on  
10      a preparative HPLC column.

#### Administration of CD40-L Compositions

The present invention provides therapeutic compositions comprising an effective amount of CD40-L in a suitable diluent or carrier and methods of treating mammals using the compositions. For therapeutic use, purified CD40-L or a  
15      biologically active analog thereof is administered to a patient, preferably a human, for treatment in a manner appropriate to the indication. Thus, for example, CD40-L pharmaceutical compositions (for example, in the form of a soluble extracellular domain, or a fragment thereof) which is administered to achieve a desired therapeutic effect can be given by bolus injection, continuous infusion, sustained release from  
20      implants, or other suitable technique. Typically, a CD40-L therapeutic agent will be administered in the form of a pharmaceutical composition comprising purified CD40-L polypeptide in conjunction with physiologically acceptable carriers, excipients or diluents. Such carriers will be nontoxic to patients at the dosages and concentrations employed. Ordinarily, the preparation of such compositions entails combining a CD40-L polypeptide with buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates  
25      including glucose, sucrose or dextrans, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with conspecific serum albumin are exemplary appropriate diluents. CD40-L sense or  
30      antisense oligonucleotides may be administered *in vivo* by administering an effective amount of a vector containing a nucleic acid sequence that encodes an effective antisense or sense oligonucleotide. Additionally, CD40-L sense or antisense oligonucleotides may be administered *ex vivo* by removing cells containing CD40-L DNA or mRNA from an individual, incorporating an antisense or sense oligonucleotide into the cells using gene transfer techniques, and re-infusing the cells into the individual.  
35

The following examples are intended to illustrate particular embodiments and not limit the scope of the invention.

### EXAMPLE 1

5 This example describes construction of a CD40/Fc DNA construct to express a soluble CD40/Fc fusion protein for use in detecting cDNA clones encoding a CD40 ligand. The cDNA sequence of the extracellular region or ligand binding domain of complete CD40 human receptor sequence was obtained using polymerase chain reaction (PCR) techniques, and is based upon the sequence published in Stamenkovic et al.,  
10 *supra*. A CD40 plasmid (CDM8) was used as a template for PCR amplification. CDM8 is described in Stamenkovic et al. and was obtained from the authors. A PCR technique (Sarki et al., *Science* 239:487, 1988) was employed using 5' (upstream) and 3' (downstream) oligonucleotide primers to amplify the DNA sequences encoding CD40 extracellular ligand binding domain. Upstream oligonucleotide primer 5'-  
15 CCGTCGACCACTGGTTCGTCTGCC -3' (SEQ ID NO:5) introduces a *Sal* 1 site upstream from an initiator methionine of CD40 and a downstream oligonucleotide primer 5'-ACAAGATCTGGGCTCTACGTATCTCAGCCGATCCTGGGGAC-3' (SEQ ID NO:7) that inserts amino acids Tyr Val Glu Pro Arg (SEQ ID NO:8) after amino acid 193 of CD40. Glu and Pro are the first two amino acids of a hinge region  
20 of human IgG1, and are followed by a *Bgl* II restriction site that was used to fuse the extracellular domain of CD40 to the remained of human IgG1 Fc region.

The DNA construct pDC406/CD40/Fc was transfected into the monkey kidney cell line CV-1/EBNA (ATCC CRL 10478). The pDC406 plasmid includes regulatory sequences derived from SV40, human immunodeficiency virus (HIV), and Epstein-Barr virus (EBV). The CV-1/EBNA cell line was derived by transfection of the CV-1 cell line with a gene encoding Epstein-Barr virus nuclear antigen-1 (EBNA-1) that constitutively expresses EBNA-1 driven from the human CMV intermediate-early enhancer/promoter. The EBNA-1 gene allows for episomal replication of expression vectors, such as pDC406, that contain the EBV origin of replication.

30 Once cells expressing the fusion construct were identified, large scale cultures of transfected cells were grown to accumulate supernatant from cells expressing CD40/Fc. The CD40/Fc fusion protein in supernatant fluid was purified by affinity purification. Briefly, one liter of culture supernatant containing the CD40/Fc fusion protein was purified by filtering mammalian cell supernatants (e.g., in a 0.45 $\mu$  filter) and applying filtrate to a protein A/G antibody affinity column (Schleicher and Schuell, Keene, NH) at 4°C at a flow rate of 80 ml/hr for a 1.5 cm x 12.0 cm column. The column was washed with 0.5 M NaCl in PBS (phosphate buffered saline) until free

protein could not be detected in wash buffer. Finally, the column was washed with PBS. Bound fusion protein was eluted from the column with 25 mM citrate buffer, pH 2.8, and brought to pH 7 with 500 mM Hepes buffer, pH 9.1. Silver-stained SDS gels of the eluted CD40/Fc fusion protein showed it to be > 98% pure.

5 Purified CD40/Fc fusion protein was iodinated with  $^{125}\text{I}$  using a commercially available solid phase agent (IODO-GEN, Pierce). In this procedure, 5  $\mu\text{g}$  of IODO-GEN were plated at the bottom of a 10 x 75 mm glass tube and incubated for twenty minutes at 4° C with 75  $\mu\text{l}$  of 0.1 M sodium phosphate, pH 7.4 and 20  $\mu\text{l}$  (2 mCi)  $\text{Na}^{125}\text{I}$ . The solution was then transferred to a second glass tube containing 5  $\mu\text{g}$  of  
10 CD40/Fc in 45  $\mu\text{l}$  PBS and this reaction mixture was incubated for twenty minutes at 4° C. The reaction mixture was fractionated by gel filtration on a 2 ml bed volume of Sephadex® G-25 (Sigma), and then equilibrated in RPMI 1640 medium containing 2.5% (v/v) bovine serum albumin (BSA), 0.2% (v/v) sodium azide and 20 mM Hepes, pH 7.4 binding medium. The final pool of  $^{125}\text{I}$  CD40/Fc was diluted to a working  
15 stock solution of  $1 \times 10^{-7}$  M in binding medium and stored for up to one month at 4° C without detectable loss of receptor binding activity.

Approximately 50% - 60% label incorporation was observed. Radioiodination yielded specific activities in the range of  $1 \times 10^{15}$  to  $5 \times 10^{15}$  cpm/nmole (0.42 - 2.0 atoms of radioactive iodine per molecule of protein). SDS polyacrylamide gel  
20 electrophoresis (SDS-PAGE) revealed a single labeled polypeptide consistent with expected values. The labeled fusion protein was greater than 98% trichloroacetic acid (TCA) precipitable, indicating that the  $^{125}\text{I}$  was covalently bound to the protein.

#### EXAMPLE 2

25 This example describes selection of a cell line putatively expressing CD40-L. Several cell lines were screened using the radioiodinated CD40/Fc fusion protein described in Example 1. Briefly, quantitative binding studies were performed according to standard methodology, and Scatchard plots were derived for the various cell lines. A clonal cell line (EL4, ATCC Catalog TIP 39) a murine thymoma cell line  
30 was identified and sorted. Prior to sorting, EL-4 cells were found to express approximately 450 molecules of CD40-L per cell. The seventh sort cells were called EL-40.7 and were grown and found to express approximately 10,000 molecules of CD40-L per cell. Lastly, the ninth sort cells were called EL-40.9 and were grown and found to express approximately 15,000 molecules of CD40-L per cell.

EXAMPLE 3

This example describes preparation of a cDNA library for expression cloning of murine CD40-L. The library was prepared from a fifth sorted clone of a mouse thymoma cell line EL-4 (ATCC TIB 39), called EL-40.5. EL-40.5 cells were EL4 cells sorted five times with biotinylated CD40/Fc fusion protein in a FACS (fluorescence activated cell sorter). A cDNA library was made from RNA obtained from EL-40.5 cells essentially as described in US Patent 4,968,607, the disclosure of which is incorporated by reference herein. Briefly, a cDNA library was constructed by reverse transcription of poly (A)<sup>+</sup> mRNA isolated from the total RNA extracted from the EL-40.5 cell line. The library construction technique was substantially similar to that described by Ausubel et al., eds., *Current Protocols In Molecular Biology*, Vol. 1, (1987). Poly (A)<sup>+</sup> mRNA was isolated by oligo dT cellulose chromatography and double-stranded cDNA was made substantially as described by Gubler et al., *Gene* 25:263, 1983. Poly(A)<sup>+</sup> mRNA fragments were converted to RNA-cDNA hybrids by reverse transcriptase using random hexanucleotides as primers. The RNA-cDNA hybrids were then converted into double-stranded cDNA fragments using RNAase H in combination with DNA polymerase I. The resulting double-stranded cDNA was blunt-ended with T4 DNA polymerase.

20 *Sal I* adaptors

5' - TCG ACT GGA ACG AGA CGA CCT GCT -3'  
                  GA CCT TGC TCT GCT GGA CGA - 5'

were ligated to 5' ends of resulting blunt-ended cDNA, as described in Haymerle et al., *Nucleic Acids Res.* 14:8615, 1986. Non-ligated adaptors were removed by gel 25 filtration chromatography at 68°C. This left 24 nucleotide non-self-complementary overhangs on cDNA. The same procedure was used to convert 5' *Sal I* ends of the mammalian expression vector pDC406 to 24 nucleotide overhangs complementary to those added to cDNA. Optimal proportions of adaptored vector and cDNA were ligated in the presence of T4 polynucleotide kinase. Dialyzed ligation mixtures were 30 electroporated into *E. coli* strain DH5 $\alpha$  and transformants selected on ampicillin plates.

Plasmid DNA was isolated from pools consisting of approximately 2,000 clones of transformed *E. coli* per pool. The isolated DNA was transfected into a sub-confluent layer of CV1-EBNA cells using DEAE-dextran followed by chloroquine treatment substantially according to the procedures described in Luthman et al., *Nucl. Acids Res.* 11:1295, 1983 and McCutchan et al., *J. Natl. Cancer Inst.* 41:351, 1986.

CV1-EBNA cells were maintained in complete medium (Dulbecco's modified Eagles' media containing 10% (v/v fetal calf serum, 50 U/ml penicillin, 50 U/ml streptomycin, and 2 mM L-glutamine) and were plated to a density of approximately 2

29

x 10<sup>5</sup> cells/well in single-well chambered slides (Lab-Tek). The slides were pre-treated with 1 ml human fibronectin (10 µg/ml PBS) for 30 minutes followed by a single washing with PBS. Media was removed from adherent cells growing in a layer and replaced with 1.5 ml complete medium containing 66.6 µM chloroquine sulfate. About 5 0.2 ml of a DNA solution (2 µg DNA, 0.5 mg/ml DEAE-dextran in complete medium containing chloroquine) was added to the cells and the mixture was incubated at 37°C for about five hours. Following incubation, media was removed and the cells were shocked by addition of complete medium containing 10% DMSO (dimethylsulfoxide) for 2.5 - 20 minutes. Shocking was followed by replacement of the solution with fresh 10 complete medium. The cells were grown in culture for two to three days to permit transient expression of the inserted DNA sequences. These conditions led to a 30% to 80% transfection frequency in surviving CV1-EBNA cells.

#### EXAMPLE 4

15 This example describes screening of the expression cloning library made in Example 3 with a labeled CD40/Fc fusion protein made in Example 1. After 48 - 72 hours, transfected monolayers of CV1-EBNA cells made in Example 3 were assayed by slide autoradiography for expression of CD40-L using radioiodinated CD40/Fc fusion protein as prepared in Example 1. Transfected CV1-EBNA cells were washed 20 once with binding medium (RPMI 1640 containing 25 mg/ml bovine serum albumin (BSA), 2 mg/ml sodium azide, 20 mM Hepes pH 7.2, and 50 mg/ml nonfat dry milk) and incubated for 2 hours at 4°C ml in binding medium containing 1 x 10<sup>-9</sup> M <sup>125</sup>I-CD40/Fc fusion protein. After incubation, cells in the chambered slides were washed three times with binding buffer, followed by two washes with PBS, (pH 7.3) to 25 remove unbound radiolabeled fusion protein.

The cells were fixed by incubating in 10% gluteraldehyde in PBS (30 minutes at room temperature), washed twice in PBS and air-dried. The slides were dipped in Kodak GTNB-2 photographic emulsion (6x dilution in water) and exposed in the dark for two to four days days at room temperature in a light-proof box. The slides were 30 developed in Kodak D19 developer, rinsed in water and fixed in Agfa G433C fixer. The slides were individually examined under a microscope at 25-40x magnification. Positive slides showing cells expressing CD40-L were identified by the presence of autoradiographic silver grains against a light background.

One pool containing approximately 2000 individual clones was identified as 35 potentially positive for binding the CD40/Fc fusion protein. The pool was titered and plated to provide plates containing approximately 200 colonies each. Each plate was scraped to provide pooled plasmid DNA for transfection into CV1-EBNA cells

IP

6

7

6

3

30

according to the same procedure described above. The smaller pools were screened by slide autoradiography as described previously. One of the smaller pools contained clones that were positive for CD40-L as indicated by the presence of an expressed gene product capable of binding to the CD40/Fc fusion protein.

5 The positive smaller pool was titered and plated to obtain individual colonies. Approximately 400 individual colonies were picked and inoculated into culture medium in individual wells of 96-well plates. Cultures were mixed by pooling rows and columns and the mixed cultures were used to prepare DNA for a final round of transfection and screening. An intersection of a positive row and a positive column indicated a potential positive colony. Ten potential positive colonies (i.e., candidate clones) were identified. DNA was isolated from each candidate clone, retransfected and rescreened. Five candidate clones were positive by binding to CD40/Fc. All five positive candidate clones contained a cDNA insert of 1468 nucleotides, as determined by dideoxynucleotide sequencing. The cDNA coding region of the CD40-L clone corresponds to the sequence of Figure 1 and SEQ ID NO:1.

10 A cloning vector containing murine CD40-L sequence, designated pDC406-mCD40-L, was deposited with the American Type Culture Collection, Rockville, MD (ATCC) on December 6, 1991, under accession number 68872. The nucleotide sequence and predicted amino acid sequence of this clone are illustrated in SEQ ID NO:1 and in Figure 1.

#### EXAMPLE 5

15 This example illustrates a cross-species hybridization technique which was used to isolate a human CD40-L homolog using a probe designed from the sequence of murine CD40-L. A murine CD40-L probe was produced by excising the coding region from murine CD40-L clone pDC406-CD40-L (nucleotide 13 through 793) and <sup>32</sup>P-labeling the fragment using random primers (Boehringer-Mannheim).

20 A human peripheral blood lymphocyte (PBL) cDNA library was constructed in a  $\lambda$  phage vector using  $\lambda$ gt10 arms and packaged *in vitro* using a commercially available kit (Gigapak<sup>®</sup> Stratagene, San Diego, CA) according to the manufacturer's instructions. The PBL cells were obtained from normal human volunteers and treated with 10 ng/ml of OKT3 (an anti-CD3 antibody), and 10 ng/ml of human IL-2 (Immunex, Seattle, WA) for six days. The PBL cells were washed and stimulated with 500 ng/ml ionomycin (Calbiochem) and 10 ng/ml PMA (Sigma) for four hours.

25 Messenger RNA and cDNA were obtained from the stimulated PBL cells and packaged into  $\lambda$ gt10 phage vectors (Gigapak<sup>®</sup> Stratagene) according to manufacturer's instructions.

31

The murine probe was hybridized to phage cDNA in 6 X SSC (15 mM trisodium citrate, and 165 mM sodium chloride), 1 X Denhardt's solution, 2 mM EDTA, 0.5% NP40 at 63°C overnight. Hybridization was followed by extensive washing in 3 X SSC, 0.1% SDS at approximately 55°C for three hours. Specific bands were visualized by autoradiography.

5 A cloning vector containing human CD40-L sequence, designated hCD40-L, was deposited with the American Type Culture Collection, Rockville, MD (ATCC) on December 6, 1991, under accession number 68873. The nucleotide sequence and predicted amino acid sequence of this clone are illustrated SEQ ID NO:11 and in Figure  
10 2.

#### EXAMPLE 6

This example illustrates the expression of membrane-bound murine CD40-L in CV1-EBNA cells. Murine CD40-L cDNA in HAVEO vector or empty HAVEO vector  
15 were transfected into CV1 EBNA cells using standard techniques, such as those described in McMahan et al. et al. *EMBO J.* 10:2821, 1991 and in Example 3 herein. Briefly, CV1 EBNA cells were plated at a density of  $2 \times 10^6$  cells per 10 cm dish in 10 ml of Dulbecco's Minimal Essential Medium supplemented with 10% fetal calf serum  
20 (Medium). The cells were allowed to adhere overnight at 37°C. The Medium was replaced with 1.5 ml of Medium containing 66.7  $\mu$ M chloroquine and a DNA mixture containing 5  $\mu$ g of cDNA encoding mCD40-L. Medium containing 175  $\mu$ l, and 25  $\mu$ l of DEAE dextran (4 mg/ml in PBS) was also added to the cells. The cells and cDNA  
25 were incubated at 37°C for 5 hours. The cDNA mixture was removed and the cells were shocked with 1 ml of fresh Medium containing 10% DMSO for 2.5 min. The Medium was replaced with fresh Medium and the cells were grown for at least 3 days.

#### EXAMPLE 7

This example illustrates the preparation of monoclonal antibodies to CD40-L. Preparations of purified murine CD40-L or human CD40-L are prepared by COS cell  
30 expression and CD40/Fc affinity purification as described herein. Purified CD40-L can generate monoclonal antibodies against CD40-L using conventional techniques, for example, those techniques described in U.S. Patent 4,411,993. Briefly, mice are immunized with CD40-L as an immunogen emulsified in complete Freund's adjuvant, and injected in amounts ranging from 10-100  $\mu$ g subcutaneously or intraperitoneally.  
35 Ten to twelve days later, the immunized animals are boosted with additional CD40-L emulsified in incomplete Freund's adjuvant. Mice are periodically boosted thereafter on a weekly to bi-weekly immunization schedule. Serum samples are periodically taken.

by retro-orbital bleeding or tail-tip excision for testing by dot blot assay or ELISA (Enzyme-Linked Immunosorbent Assay), for CD40-L antibodies.

Following detection of an appropriate antibody titer, positive animals are provided one last intravenous injection of CD40-L in saline. Three to four days later, 5 the animals are sacrificed, spleen cells harvested, and spleen cells are fused to a murine myeloma cell line (e.g., NS1 or Ag 8.653). Fusions generate hybridoma cells, which are plated in multiple microtiter plates in a HAT (hypoxanthine, aminopterin and thymidine) selective medium to inhibit proliferation of non-fused cells, myeloma hybrids, and spleen cell hybrids.

10 The hybridoma cells are screened by ELISA for reactivity against purified CD40-L by adaptations of the techniques disclosed in Engvall et al., *Immunochem.* 8:871, 1971 and in U.S. Patent 4,703,004. Positive hybridoma cells can be injected intraperitoneally into syngeneic BALB/c mice to produce ascites containing high concentrations of anti-CD40-L monoclonal antibodies. Alternatively, hybridoma cells 15 can be grown *in-vitro* in flasks or roller bottles by various techniques. Monoclonal antibodies produced in mouse ascites can be purified by ammonium sulfate precipitation, followed by gel exclusion chromatography. Alternatively, affinity chromatography based upon binding of antibody to protein A or protein G can also be used, as can affinity chromatography based upon binding to CD40-L.

20

#### EXAMPLE 8

This example illustrates anti-allergy therapeutic effects of sCD40 and CD40/Fc fusion protein. Soluble CD40 and CD40/Fc were tested for their ability to inhibit IL-4 (5 ng/ml) induced IgE secretion in a two donor MLC system. The data from three 25 experiments are presented in Table 1.

Table 1

Addition	IgE (ng/ml)	
	Exp. 1	Exp. 2
Exp. 3		
30 medium	<0.1	<0.1
<0.1		
IL-4	24	47
IL-4 + sCD40 (0.1 µg/ml)	19	nd
IL-4 + sCD40 (0.3 µg/ml)	14	29
35 IL-4 + sCD40 (1 µg/ml)	10	24
IL-4 + sCD40 (3 µg/ml)	7	19
IL-4 + IL-7R/Fc (10 µg/ml)	21	nd
		58

ip

6

7

8

9

IgE levels were measured after 12 days in culture by an ELISA procedure. Briefly, flat-bottomed 96-well microtiter plates (Corning) were coated with mouse mAb anti-human IgE (Zymed) at 1:500 dilution in PBS (phosphate buffered saline). After washing 3X, a blocking step was performed using 5% non-fat dried milk, followed by titration of human IgE standards or test supernatants. After washing 3X, biotinylated goat anti-human IgE (Kirkegaard and Perry) was added at a 1:500 dilution. This was followed by further washing and then addition of streptavidin-HRP (Zymed) at a 1:500 dilution. After further washing, the reaction was developed using TMB substrate (Kirkegaard and Perry) and absorbance measured at 520 nm. All washing steps were carried out in PBS plus 0.05% Tween. All incubation steps were performed at volumes of 100  $\mu$ l/well for one hour at room temperature. The sensitivity of this assay is 100 pg/ml.

#### EXAMPLE 9

15. This example illustrates the effects of sCD40 and CD40/Fc fusion protein to inhibit soluble CD23 shedding from IL-4 (5 ng/ml) stimulated B cells. Soluble CD40 and CD40/Fc were tested for their ability to inhibit IL-4-induced sCD23 shedding in a two donor MLC system. The data from three experiments are presented in Table 2.

Table 2

	Addition	sCD23 (ng/ml)					
		Exp. 1		Exp. 2		Exp. 3	
		day 6	day 12	day 6	day 12	day 6	day
12							
	E <sup>+</sup> + medium	55	<0.5	24	10	10	5
25	+ IL-4	115	55	96	62	44	27
	+ IL-4 + sCD40 (1 $\mu$ g/ml)	nd	nd	88	36	38	9
	+ IL-4 + sCD40 (3 $\mu$ g/ml)	97	4	82	31	40	4
	+ IL-4 + sCD40 (10 $\mu$ g/ml)	nd	nd	72	28	nd	nd
	+ IL-4 + IL-7R/Fc (3 $\mu$ g/ml)	111	48	103	67	40	22
30							
	PBM + medium	12	<0.5	15	5	3	10
	+ IL-4	39	255	47	22	48	26
	+ IL-4 + sCD40 (1 $\mu$ g/ml)	nd	nd	44	18	46	18
	+ IL-4 + sCD40 (3 $\mu$ g/ml)	24	6	37	11	45	12
35	+ IL-4 + sCD40 (10 $\mu$ g/ml)	nd	nd	28	5	nd	nd
	+ IL-4 + IL-7R/Fc (3 $\mu$ g/ml)	35	26	43	20	50	23

Soluble CD23 levels were measured after 6 and 12 days in culture by a commercial sCD23 ELISA detection kit (Binding Site, San Diego, CA). The sensitivity limit was 500 pg/ml. Approximately  $1 \times 10^5$  cells per well were cultured in triplicate in round-bottomed 96-well microtiter plates (Intermountain Scientific, Bountiful UT) for 5 the indicated time in the presence or absence of additives as indicated in Table 2. The results show anti-allergy effects of sCD40. Similar studies were run with CD40/Fc (data not shown) instead of sCD40, and similar results were obtained. Accordingly, these data in Examples 8 and 9 illustrate an anti-allergy property for CD40.

10

#### EXAMPLE 10

This example illustrates B cell proliferative activity of membrane-bound murine CD40-L for human B cells. Human peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood from normal volunteers by density gradient centrifugation over Histopaque® (Sigma, St. Louis, MO). T cell-depleted preparations of cells (E-) were obtained by removing T cells by rosetting with 2-aminoethylisothiouronium bromide-treated SRBC (sheep red blood cells) and further density gradient centrifugation over Histopaque®. B cell proliferation assays were conducted with E- preparations in RPMI media with added 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a 10% CO<sub>2</sub> atmosphere. Approximately  $1 \times 10^5$  E- 15 cells per well were cultured in triplicate in flat-bottomed 96-well microtiter plates (Corning) for 7 days in the presence of transfected CV1 EBNA cells (described in Example 6). The CV1 EBNA cells were transfected with murine CD40-L cDNA or empty vector. The cells were pulsed with 1 μCi/well of tritiated thymidine (25 Ci/nmole Amersham, Arlington Heights, IL) for the final eight hours of culture. Cells 20 were harvested onto glass fiber discs with an automated cell harvester and incorporated cpm were measured by liquid scintillation spectrometry. Cells 25 were harvested onto glass fiber discs with an automated cell harvester and incorporated cpm were measured by liquid scintillation spectrometry.

Figure 4a shows a comparison of human B cell proliferation of CV1 EBNA cells transfected with empty vector (HAVEO) or with murine CD40-L cDNA in HAVEO vector. These data show that membrane-bound CD40-L stimulates human B cell proliferation in the absence of a co-mitogen. Figure 4b shows a similar 30 experiment, except that 10 ng/ml of human IL-4 was added to the cultures. In this experiment, IL-4 slightly enhances the B cell mitogenic activity of membrane-bound murine CD40-L. Figure 5 is a repeat of the experiment shown in Figure 4b. However, when the experiment was repeated, there was no evidence of IL-4 co-mitogenic 35 activity. There was repeated evidence of CD40-L mitogenic activity. Accordingly, membrane-bound CD40-L stimulates proliferation of human B cells.

35

EXAMPLE 11

This example illustrates the effect of membrane-bound murine CD40-L to stimulate IgE production and CD23 shedding from E<sup>-</sup> cells isolated in Example 10. Approximately 1 X 10<sup>5</sup> cells/well were cultured in triplicate round bottomed 96-well 5 Nunc microtiter plates (Intermountain Scientific, Bountiful UT) in Iscove's Modified Dulbecco's Medium (IMDM) plus 10% FCS in a humidified atmosphere of 10% CO<sub>2</sub>. Medium was supplemented with 50 µg/ml human transferrin (Sigma); 0.5% bovine serum albumin (Sigma) and 1 µg/ml of each of oleic, linoleic and palmitic acids (Sigma). The E<sup>-</sup> cells were cultured for 10 days in the presence of 5 ng/ml human IL-10. 4. A titration of CV1 EBNA cells transfected with murine CD40-L or empty vector were added. After ten days, culture supernatants were assayed for IgE by the ELISA procedure described in Example 8 or for CD23 shedding by the procedure described in Example 9.

Figure 6 shows a comparison of IgE production in the supernatants (in ng/ml) 15 for cultures of E<sup>-</sup> cells and CV1 EBNA cells transfected with empty vector (HAVEO) or with CD40-L. No differences were noted with up to 3000 CV1 EBNA cells, however significant IgE production resulted with the addition of 10000 or 30000 CD40-L transfected CV1 EBNA cells. As a comparison, when E<sup>-</sup> cells were incubated with medium alone, 5 ng/ml IL-4 or 5 ng/ml IL-4 plus 500 ng/ml G28-5 antibody, IgE 20 production was 4.7, 2.9 and >600 ng/ml, respectively. When CD23 shedding was measured in Figure 7, 10000 and 30000 CV1 EBNA cells transfected with CD40-L showed increased CD23 shedding when compared to empty vector control CV1 EBNA cells. As a comparison, when E<sup>-</sup> cells were incubated with medium alone, 5 ng/ml IL-4 or 5 ng/ml IL-4 plus 500 ng/ml G28-5 antibody, CD23 shedding was <0.1, 2.4 and 25 11.2 ng/ml, respectively. These data show that IgE production and CD23 shedding are both biological activities associated with membrane-bound CD40-L.

EXAMPLE 12

This example illustrates B cell proliferative activity, polyclonal immunoglobulin (Ig) production, antigen-specific antibody formation and various method for using 30 membrane-bound and soluble CD40-L in clinical applications. We obtained murine splenic B cells according to procedures described in Grabstein et al. *I supra*, Maliszewski et al. *I supra* and Maliszewski et al. *II supra*. Briefly, the mixed culture of cells was purified by T cell depletion using T cell antiserum and complement, and 35 adherent cell depletion by passage of Sephadex® G10 columns and by B cell positive selection by panning on petri dishes coated with goat anti-mouse IgM. Purified B cells were cultured in RPMI, fetal calf serum (5% for B cell proliferation assays and 20% for

plaque forming cell assays or polyclonal antibody assays), 2-mercaptoethanol, antibiotics, amino acids and pyruvate. B cell proliferation was measured according to the assay described in Example 10 and in Grabstein et al. I *supra*, Maliszewski et al. I *supra* and Maliszewski et al. II *supra*. Antigen-specific antibody formation was measured by the procedure described in Grabstein et al., *J. Mol. Cell. Immunol.* 2:199, 1986 [Grabstein et al. III]. Briefly, antigen specific antibody formation used sheep red blood cells (SRBC) as antigen (0.03% v/v) in 2.0 ml cultures of  $1 \times 10^6$  murine B cells per culture. The B cell cultures were incubated for 5 days and plaque forming cells were determined by Jerne hemolytic plaque assay as described in Grabstein et al. II *supra*. Cell counts were determined in a coulter counter. Polyclonal Ig secretion was determined by isotype-specific ELISA assays in seven day cultures of  $1 \times 10^6$  B cells per 2.0 ml culture as described in Maliszewski et al. I *supra* and Maliszewski et al. II *supra*.

The results of B cell proliferation by CV1 EBNA cells transfected with CD40-L or empty vector or 7A1 cells (a T cell helper clone) are shown in Figures 8, 10 and 12. These data show that the greatest B cell proliferation was caused by CD40-L. T cell helper cells 7A1 and 7C2 had a minimal effect on B cell proliferation.

The effects of various cells upon antigen specific antibody formation are shown in Figures 9 and 11. Figure 9 shows a comparison of plaque forming cells comparing T cell helper clone 7A1 and murine EL40.9 cells which secrete a soluble CD40-L. The EL40.9 cells seem to have an inhibitory effect upon antigen specific antibody formation. Figure 11 shows PFC (plaque forming cells) for T cell helper cells 7C2 and CV1 EBNA cells transfected with either empty vector or CD40-L. Both 7C2 cells and membrane-bound CD40-L stimulated antigen specific antibody formation (PFC). Figure 13 compares antigen specific antibody formation of CD40-L and 7A1 cells in the presence or absence of 10 ng/ml interleukin-2 (IL-2). IL-2 increased PFC for 7A1 cells but did not increase PFC caused by membrane-bound CD40-L.

Polyclonal Ig production by murine B cells was compared for stimulation or inhibition with membrane-bound CD40-L, control CV1 EBNA cells and helper-T cells 7A1 in the presence of cytokines IL-4 (10 ng/ml) and IL-5 (1:40 dilution of COS cell supernatants) or without added cytokines. The amount of IgA, IgG3, IgE, IgG2b, IgM and IgG1 are shown in Tables 3-8, respectively.

TABLE 3

IgA, ng/ml

		# CELLS	MEDIA	+IL-4+IL-5
10	CD40-L	2 X 10(5)	666.275 ± 174.444	64.639 ± 51.780
		1 X 10(5)	288.085 ± 20.773	291.831 ± 10.673
		1 X 10(4)	53.750 ± 36.531	910.072 ± 62.713
15	HAVEO	2 X 10(5)	0	628.190 ± 42.907
		1 X 10(5)	0	477.755 ± 57.478
		1 X 10(4)	0	295.640 ± 12.736
20	7A1 (2C11)	1 X 10(6)	0	2177.549 ± 377.052
		2 X 10(5)	0	646.898 ± 86.325
		1 X 10(5)	0	480.671 ± 40.011
25	MEDIA LPS		0	458.152 ± 77.258
			88.531 ± 31.248	132.336 ± 51.356

TABLE 4

IgG3, ng/ml

		# CELLS	MEDIA	+IL-4+IL-5
30	CD40-L	2 X 10(5)	108.427 ± 14.359	0
		1 X 10(5)	118.079 ± 8.021	46.535 ± 9.899
		1 X 10(4)	127.591 ± 6.268	467.023 ± 78.276
35	HAVEO	2 X 10(5)	0	29.773 ± 5.224
		1 X 10(5)	11.205 ± 4.434	66.323 ± 8.673
		1 X 10(4)	26.389 ± 10.221	34.671 ± 12.975
40	7A1 (2C11)	1 X 10(6)	33.420 ± 9.972	820.856 ± 39.442
		2 X 10(5)	0	436.074 ± 59.332
		1 X 10(5)	0	239.760 ± 45.978
45	MEDIA LPS		21.808 ± 7.107	64.773 ± 13.924
			816.697 ± 43.553	103.720 ± 11.883

38

TABLE 5

IgE, ng/ml

		# CELLS	MEDIA	+IL-4+IL-5
10	CD40-L	2 X 10(5)	0	64.144 ± 4.979
		1 X 10(5)	0	83.493 ± 9.093
		1 X 10(4)	0	461.155 ± 60.514
15	HAVEO	2 X 10(5)	0	0
		1 X 10(5)	0	4.208 ± .527
		1 X 10(4)	0	0
20	7A1 (2C11)	1 X 10(6)	0	208.091 ± 8.090
		2 X 10(5)	0	32.530 ± 0.723
		1 X 10(5)	0	15.889 ± 2.947
25	MEDIA		0	12.602 ± 1.460
		LPS	0	408.355 ± 9.764

TABLE 6

IgG2b, ng/ml

		# CELLS	MEDIA	+IL-4+IL-5
30	CD40-L	2 X 10(5)	0	0
		1 X 10(5)	0	6.230 ± .285
		1 X 10(4)	0	47.414 ± .241
35	HAVEO	2 X 10(5)	0	7.001 ± 2.358
		1 X 10(5)	0	6.230 ± 2.285
		1 X 10(4)	0	9.620 ± 2.650
40	7A1 (2C11)	1 X 10(6)	0	189.343 ± 2.837
		2 X 10(5)	0	22.431 ± 6.835
		1 X 10(5)	0	7.207 ± 1.580
45	MEDIA		0	7.422 ± 1.602
		LPS	0	33.291 ± 3.183

50

6

3

39

TABLE 7

IgM,  $\mu$ g/ml

		# CELLS	MEDIA	+IL-4+IL-5
10	CD40-L	2 X 10(5)	1.805 $\pm$ 0.639	0.439 $\pm$ 0.184
		1 X 10(5)	2.237 $\pm$ 0.583	5.878 $\pm$ 0.858
		1 X 10(4)	2.293 $\pm$ 0.595	96.730 $\pm$ 13.009
15	HAVEO	2 X 10(5)	0	10.890 $\pm$ 2.126
		1 X 10(5)	0	13.303 $\pm$ 0.993
		1 X 10(4)	0.624 $\pm$ 0.178	22.538 $\pm$ 2.304
20	7A1 (2C11)	1 X 10(6)	0.769 $\pm$ 0.124	104.857 $\pm$ 17.463
		2 X 10(5)	0.142 $\pm$ 0.052	27.016 $\pm$ 1.706
		1 X 10(5)	0.126 $\pm$ 0.048	13.070 $\pm$ 0.600
25	MEDIA LPS		0.231 $\pm$ 0.057	36.809 $\pm$ 2.860
			53.302 $\pm$ 9.668	41.974 $\pm$ 6.158

TABLE 8

IgG1, ng/ml

		# CELLS	MEDIA	+IL-4+IL-5
30	CD40-L	2 X 10(5)	0	130.185 $\pm$ 24.547
		1 X 10(5)	0	310.588 $\pm$ 1.261
		1 X 10(4)	0	270.727 $\pm$ 17.511
35	HAVEO	2 X 10(5)	0	187.668 $\pm$ 57.730
		1 X 10(5)	0	43.320 $\pm$ 49.770
		1 X 10(4)	0	1363.464 $\pm$ 45.841
40	7A1 (2C11)	1 X 10(6)	0	145.652 $\pm$ 136.070
		2 X 10(5)	0	365.563 $\pm$ 24.276
		1 X 10(5)	0	449.475 $\pm$ 101.012
45	MEDIA LPS		0	133.660 $\pm$ 386.231
			0	246.213 $\pm$ 21.526

These data indicate that the interaction of CD40 with its ligand is the principal molecular interaction responsible for T cell contact dependent induction of B cell growth and differentiation to both antigen-specific antibody production and polyclonal Ig secretion. As such, these data suggest that antagonists of this interaction, by soluble CD40, CD40/Fc fusion protein and possibly soluble CD40-L (monomeric), will

significantly interfere with development of antibody responses. Therefore clinical situations where CD40, CD40/Fc fusion proteins and soluble CD40-L include allergy, lupus, rheumatoid arthritis, insulin dependent diabetes mellitus, and any other diseases where autoimmune antibody or antigen/antibody complexes are responsible for clinical pathology of the disease. Moreover, membrane-bound CD40-L or oligomeric soluble CD40-L will be useful to stimulate B cell proliferation and antibody production. As such, these forms of CD40-L are most useful for vaccine adjuvants and as a stimulating agent for mAb secretion from hybridoma cells.

10

#### EXAMPLE 13

This example illustrates the effect of membrane-bound CD40-L upon proliferation of and IgE secretion from peripheral blood mononuclear cells (E<sup>-</sup>). E<sup>-</sup> cells were obtained according to the procedure described in Example 10 and incubated for 7 or 10 days in the presence of CV1 EBNA cells transfected with empty vector or 15 mCD40-L cDNA. Additionally, CD40/Fc fusion protein (described in Example 1) or TNF Receptor/Fc fusion protein (described in WO 91/03553) was added to some of the preparations as indicated in Figure 14. IgE secretion was measured according to the procedure described in Example 8 and B cell proliferation was measured according to the procedure described in Example 10.

20

The results for B cell proliferation and IgE secretion are shown in Figure 14 for five different concentrations of transfected CV1 EBNA cells. Both B cell proliferation and IgE secretion were increased in the presence of membrane-bound CD40-L. Addition of CD40/Fc fusion protein ablated both B cell proliferation and IgE secretion. The TNF Receptor/Fc fusion protein had no effect. As a comparison for IgE secretion, 25 addition of IL-4 as a control agent (without transfected CV1 EBNA cells) produced no IgE in this assay and addition of IL-4 plus G28-5 anti-CD40 mAb resulted in 29.7 ng/ml IgE in this assay.

#### EXAMPLE 14

30 This example describes construction of a CD40-L/Fc DNA construct to express a soluble CD40-L/Fc fusion protein referred to as CD40-L/FC2 construct. DNA encoding CD40-L/FC2 comprises sequences encoding a leader (or signal) peptide, an eight amino acid hydrophilic sequence described by Hopp et al. (Hopp et al., *Bio/Technology* 6:1204, 1988; referred to as Flag<sup>®</sup>), a suitable Fc region of an 35 immunoglobulin, a [Gly4Ser]<sub>3</sub> repeat sequence (described in U.S. Patent 5,073,627, which is incorporated by reference herein) or other suitable linker sequence, and the extracellular region of human CD40-L from amino acid 50 to amino acid 261 (SEQ ID

41

NO:11). A pDC406 expression vector containing a leader sequence, Flag®, and human IgG<sub>1</sub> Fc is prepared using conventional techniques of enzyme cutting and ligation of fragments encoding a leader sequence, Flag®, and human IgG<sub>1</sub> Fc, and restricted with *Nsi* 1 and *Not* 1.

5 A PCR technique (Sarki et al., *Science* 239:487, 1988) was employed using 5' (upstream) and 3' (downstream) oligonucleotide primers to amplify the DNA sequences encoding CD40 extracellular ligand binding domain from a cloning vector containing human CD40-L (ATCC 68873; SEQ ID NO: 11) to form a PCR fragment. The upstream oligonucleotide primer (SEQ ID NO:13) introduced a *Nsi* 1 site upstream  
10 from a linker sequence ([Gly<sub>4</sub>Ser]<sub>3</sub>SerSer), which was followed by 21 nucleotides of the extracellular domain of CD40-L (amino acids 51 through 57 of SEQ ID NO: 11). A downstream oligonucleotide primer (SEQ ID NO:14) introduced a *Not* 1 site just downstream of the termination codon of the CD40-L. The PCR fragment was then ligated into the pDC406 expression vector containing a leader sequence, Flag®, and  
15 human IgG<sub>1</sub> Fc. The nucleotide and predicted amino acid sequence of CD40-L/FC2 are presented in SEQ ID NO:15 and SEQ ID NO:16. The resultant DNA construct (CD40-L/FC2) was transfected into the monkey kidney cell line CV-1/EBNA (ATCC CRL 10478). The construct encoded a soluble CD40-L capable of binding CD40, as evidenced by binding observed in fluorescence-activated cell sorting (FACS) analysis  
20 using cells that express CD40.

Large scale cultures of human embryonic kidney 293 cells (ATCC CRL 1573) transfected with the construct encoding CD40-L/FC2 were grown to accumulate supernatant containing CD40-L/FC2. The 293 cell line, a permanent line of primary human embryonal kidney transformed by human adenovirus 5 DNA, permits  
25 expression of recombinant proteins ligated into the pCD406 vector. The CD40-L/FC2 fusion protein in supernatant fluid was purified by affinity purification. Briefly, culture supernatant containing the CD40-L/FC2 fusion protein was purified by filtering mammalian cell supernatants (e.g., in a 0.45μ filter) and applying filtrate to an antibody affinity column comprising biotinylated goat anti-human IgG (Jackson Immunoresearch  
30 Laboratories, Inc., Westgrove, PA, USA) coupled to Streptavidin-agarose (Pierce Chemical, Rockford, IL, USA) at 4°C, at a flow rate of approximately 60 to 80 ml/hr for a 1.5 cm x 12.0 cm column. The column was washed with approximately 20 column volumes of PBS (phosphate buffered saline), until free protein could not be detected in wash buffer. Bound fusion protein was eluted from the column with 12.5 mM citrate buffer, 75 mM NaCl, pH 2.8, and brought to pH 7 with 500 mM Hepes buffer, pH 9.1. The purified, oligomeric CD40-L/FC2 peptide induced human B cell proliferation in the absence of any co-stimuli, and (in conjunction with the appropriate  
35

1P

6

7

8

9

cytokine) resulted in the production of IgG, IgE, IgA and IgM, as described in Example 12 for membrane-bound CD40-L.

#### EXAMPLE 15

5 This example describes construction of a CD40-L DNA construct to express a soluble CD40-L fusion protein referred to as trimeric CD40-L. Trimeric CD40-L contains a leader sequence, a 33 amino acid sequence referred to as a "leucine zipper" (SEQ ID NO:17), and an eight amino acid hydrophilic sequence described by Hopp et al. (Hopp et al., *Bio/Technology* 6:1204,1988; referred to as Flag<sup>®</sup>), followed by the 10 extracellular region of human CD40-L from amino acid 50 to amino acid 261 (SEQ ID NO:11). The utility of the leader and the Flag<sup>®</sup> sequences have been described in the Detailed Description. The 33 amino acid sequence presented in SEQ ID NO:17 trimerizes spontaneously in solution. Fusion proteins comprising this 33 amino acid sequence are thus expected to form trimers or multimers spontaneously.

15 The construct is prepared by synthesizing oligonucleotides representing a leader sequence, the 33 amino acid sequence described above, and the Flag<sup>®</sup> sequence, then ligating the final product to a DNA fragment encoding amino acids 51 through 261 of SEQ ID NO:11, prepared as described in Example 14.

20 The resulting ligation product in expression vector pDC406 was transfected into the monkey kidney cell line CV-1/EBNA (ATCC CRL 10478). The pDC406 plasmid includes regulatory sequences derived from SV40, human immunodeficiency virus (HIV), and Epstein-Barr virus (EBV). The CV-1/EBNA cell line was derived by transfection of the CV-1 cell line with a gene encoding Epstein-Barr virus nuclear antigen-1 (EBNA-1) that constitutively expresses EBNA-1 driven from the human 25 CMV intermediate-early enhancer/promoter. The EBNA-1 gene allows for episomal replication of expression vectors, such as pDC406, that contain the EBV origin of replication.

Once cells expressing the fusion construct are identified, large scale cultures of 30 transfected cells are grown to accumulate supernatant from cells expressing trimeric CD40-L. The trimeric CD40-L fusion protein in supernatant fluid is purified by affinity purification substantially as described in U.S. Patent 5,011,912. Silver-stained SDS gels of the eluted CD40-L fusion protein can be prepared to determine purity.

35

SEQUENCE LISTING

40 (1) GENERAL INFORMATION:

40 (i) APPLICANT: ARMITAGE, RICHARD

43

FANSLOW, WILLIAM  
SPRIGGS, MELANIE

5 (ii) TITLE OF INVENTION: NOVEL CYTOKINE

10 (iii) NUMBER OF SEQUENCES: 17

15 (iv) CORRESPONDENCE ADDRESS:  
(A) ADDRESSEE: IMMUNEX CORPORATION  
(B) STREET: 51 UNIVERSITY STREET  
(C) CITY: SEATTLE  
(D) STATE: WASHINGTON  
(E) COUNTRY: USA  
(F) ZIP: 98101

20 (v) COMPUTER READABLE FORM:  
(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

25 (vi) CURRENT APPLICATION DATA:  
(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

30 (viii) ATTORNEY/AGENT INFORMATION:  
(A) NAME: OSTER, JEFFREY B.  
(B) REGISTRATION NUMBER: 32585  
(C) REFERENCE/DOCKET NUMBER: 2802

35 (ix) TELECOMMUNICATION INFORMATION:  
(A) TELEPHONE: 2065870430  
(B) TELEFAX: 2065870606

40 (2) INFORMATION FOR SEQ ID NO:1:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 783 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: cDNA

50 (iii) HYPOTHETICAL: NO

55 (iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:  
(A) ORGANISM: MOUSE

60 (vii) IMMEDIATE SOURCE:  
(B) CLONE: CD40-L

(ix) FEATURE:  
(A) NAME/KEY: CDS  
(B) LOCATION: 1..783.

44

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATG ATA GAA ACA TAC AGC CAA CCT TCC CCC AGA TCC GTG GCA ACT GGA  
 48  
 5 Met Ile Glu Thr Tyr Ser Gln Pro Ser Pro Arg Ser Val Ala Thr Gly  
 1 5 10 15

CTT CCA GCG AGC ATG AAG ATT TTT ATG TAT TTA CTT ACT GTT TTC CTT  
 96  
 10 Leu Pro Ala Ser Met Lys Ile Phe Met Tyr Leu Leu Thr Val Phe Leu  
 20 25 30

ATC ACC CAA ATG ATT GGA TCT GTG CTT TTT GCT GTG TAT CTT CAT AGA  
 144  
 15 Ile Thr Gln Met Ile Gly Ser Val Leu Phe Ala Val Tyr Leu His Arg  
 35 40 45

AGA TTG GAT AAG GTC GAA GAG GAA GTA AAC CTT CAT GAA GAT TTT GTA  
 192  
 20 Arg Leu Asp Lys Val Glu Glu Val Asn Leu His Glu Asp Phe Val  
 50 55 60

TTC ATA AAA AAG CTA AAG AGA TGC AAC AAA GGA GAA GGA TCT TTA TCC  
 240  
 25 Phe Ile Lys Lys Leu Lys Arg Cys Asn Lys Gly Glu Gly Ser Leu Ser  
 65 70 75 80

TTG CTG AAC TGT GAG GAG ATG AGA AGG CAA TTT GAA GAC CTT GTC AAG  
 288  
 30 Leu Leu Asn Cys Glu Glu Met Arg Arg Gln Phe Glu Asp Leu Val Lys  
 85 90 95

GAT ATA ACG TTA AAC AAA GAA GAG AAA AAA GAA AAC AGC TTT GAA ATG  
 336  
 35 Asp Ile Thr Leu Asn Lys Glu Glu Lys Lys Glu Asn Ser Phe Glu Met  
 100 105 110

CAA AGA GGT GAT GAG GAT CCT CAA ATT GCA GCA CAC GTT GTA AGC GAA  
 384  
 40 Gln Arg Gly Asp Glu Asp Pro Gln Ile Ala Ala His Val Val Ser Glu  
 115 120 125

GCC AAC AGT AAT GCA GCA TCC GTT CTA CAG TGG GCC AAG AAA GGA TAT  
 432  
 45 Ala Asn Ser Asn Ala Ala Ser Val Leu Gln Trp Ala Lys Lys Gly Tyr  
 130 135 140

TAT ACC ATG AAA AGC AAC TTG GTA ATG CTT GAA AAT GGG AAA CAG CTG  
 480  
 50 Tyr Thr Met Lys Ser Asn Leu Val Met Leu Glu Asn Gly Lys Gln Leu  
 145 150 155 160

ACG GTT AAA AGA GAA GGA CTC TAT TAT GTC TAC ACT CAA GTC ACC TTC  
 528  
 55 Thr Val Lys Arg Glu Gly Leu Tyr Tyr Val Tyr Thr Gln Val Thr Phe  
 165 170 175

TGC TCT AAT CGG GAG CCT TCG AGT CAA CGC CCA TTC ATC GTC GGC CTC  
 576  
 60 Cys Ser Asn Arg Glu Pro Ser Ser Gln Arg Pro Phe Ile Val Gly Leu  
 180 185 190

P

□

□

6

3

45

TGG CTG AAG CCC AGC AGT GGA TCT GAG AGA ATC TTA CTC AAG GCG GCA  
 624  
 5 Trp Leu Lys Pro Ser Ser Gly Ser Glu Arg Ile Leu Leu Lys Ala Ala  
 195 200 205  
 AAT ACC CAC AGT TCC TCC CAG CTT TGC GAG CAG CAG TCT GTT CAC TTG  
 672  
 10 Asn Thr His Ser Ser Gln Leu Cys Glu Gln Gln Ser Val His Leu  
 210 215 220  
 GGC GGA GTG TTT GAA TTA CAA GCT GGT GCT TCT GTG TTT GTC AAC GTG  
 720  
 15 Gly Gly Val Phe Glu Leu Gln Ala Gly Ala Ser Val Phe Val Asn Val  
 225 230 235 240  
 ACT GAA GCA AGC CAA GTG ATC CAC AGA GTT GGC TTC TCA TCT TTT GGC  
 768  
 20 Thr Glu Ala Ser Gln Val Ile His Arg Val Gly Phe Ser Ser Phe Gly  
 245 250 255  
 TTA CTC AAA CTC TG  
 783  
 25 Leu Leu Lys Leu  
 260

## (2) INFORMATION FOR SEQ ID NO:2:

30 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 260 amino acids  
 (B) TYPE: amino acid  
 (C) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

40 Met Ile Glu Thr Tyr Ser Gln Pro Ser Pro Arg Ser Val Ala Thr Gly  
 1 5 10 15

45 Leu Pro Ala Ser Met Lys Ile Phe Met Tyr Leu Leu Thr Val Phe Leu  
 20 25 30

50 Ile Thr Gln Met Ile Gly Ser Val Leu Phe Ala Val Tyr Leu His Arg  
 35 40 45

55 Arg Leu Asp Lys Val Glu Glu Val Asn Leu His Glu Asp Phe Val  
 50 55 60

60 Phe Ile Lys Lys Leu Lys Arg Cys Asn Lys Gly Glu Gly Ser Leu Ser  
 65 70 75 80

65 Leu Leu Asn Cys Glu Glu Met Arg Arg Gln Phe Glu Asp Leu Val Lys  
 85 90 95

70 Asp Ile Thr Leu Asn Lys Glu Glu Lys Lys Glu Asn Ser Phe Glu Met  
 100 105 110

75 Gln Arg Gly Asp Glu Asp Pro Gln Ile Ala Ala His Val Val Ser Glu  
 115 120 125

46

Ala Asn Ser Asn Ala Ala Ser Val Leu Gln Trp Ala Lys Lys Gly Tyr  
130 135 140

5 Tyr Thr Met Lys Ser Asn Leu Val Met Leu Glu Asn Gly Lys Gln Leu  
145 150 155 160

Thr Val Lys Arg Glu Gly Leu Tyr Tyr Val Tyr Thr Gln Val Thr Phe  
165 170 175

10 Cys Ser Asn Arg Glu Pro Ser Ser Gln Arg Pro Phe Ile Val Gly Leu  
180 185 190

15 Trp Leu Lys Pro Ser Ser Gly Ser Glu Arg Ile Leu Leu Lys Ala Ala  
195 200 205

Asn Thr His Ser Ser Ser Gln Leu Cys Glu Gln Gln Ser Val His Leu  
210 215 220

20 Gly Gly Val Phe Glu Leu Gln Ala Gly Ala Ser Val Phe Val Asn Val  
225 230 235 240

Thr Glu Ala Ser Gln Val Ile His Arg Val Gly Phe Ser Ser Phe Gly  
245 250 255

25 Leu Leu Lys Leu  
260

## 30 (2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 740 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

40 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

45 (vi) ORIGINAL SOURCE:

(A) ORGANISM: HUMAN

(vii) IMMEDIATE SOURCE:

(B) CLONE: IgG1 Fc

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGGTACCGCT AGCGTCGACA GGCCTAGGAT ATCGATACGT AGAGCCCAGA TCTTGTGACA  
60

55 AAACTCACAC ATGCCACCG TGCCCAGCAC CTGAACTCCT GGGGGGACCG TCAGTCTTCC  
120

60 TCTTCCCCCC AAAACCCAAG GACACCCTCA TGATCTCCCG GACCCCTGAG GTCACATGCG  
180

IP

□

□

5

3

47

TGGTGGTGGGA CGTGAGCCAC GAAGACCTG AGGTCAAGTT CAACTGGTAC GTGGACGGCG  
240

5 TGGAGGTGCA TAATGCCAAG ACAAAAGCCGC GGGAGGAGCA GTACAACAGC ACGTACCGGG  
300

TGGTCAGCGT CCTCACCGTC CTGCACCAGG ACTGGCTGAA TGGCAAGGAC TACAAGTGCA  
360

10 AGGTCTCCAA CAAAGCCCTC CCAGCCCCA TGCAGAAAAC CATCTCCAAA GCCAAAGGGC  
420

AGCCCCGAGA ACCACAGGTG TACACCCCTGC CCCCATCCCG GGATGAGCTG ACCAAGAAC  
480

15 AGGTCAAGCCT GACCTGCCTG GTCAAAGGCT TCTATCCCAG GCACATGCC GTGGAGTGGG  
540

AGAGCAATGG GCAGCCGGAG AACAACTACA AGACCACGCC TCCCGTGCTG GACTCCGACG  
20 600

GCTCCTTCTT CCTCTACAGC AAGCTCACCG TGGACAAGAG CAGGTGGCAG CAGGGGAACG  
660

25 TCTTCTCATG CTCCGTGATG CATGAGGCTC TGCACAACCA CTACACGCAG AAGAGCCTCT  
720

CCCTGTCTCC GGGTAAATGA  
30 740

(2) INFORMATION FOR SEQ ID NO:4:

35 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 519 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: cDNA

45 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:  
(A) ORGANISM: HUMAN

50 (vii) IMMEDIATE SOURCE:  
(B) CLONE: CD40 EXTRACELLULAR REGION

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

55 CAGAACCACC CACTGCATGC AGAGAAAAAC AGTACCTAAT AAACAGTCAG TGCTGTTCTT  
60

TGTGCCAGCC AGGACAGAAA CTGGTGAGTG ACTGCACAGA GTTCACTGAA ACGGAATGCC  
120

60 TTCCCTGCGG TGAAAGCGAA TTCTAGACA CCTGGAACAG AGAGACACAC TGCCACCAGC  
180

48

ACAAATACTG CGACCCCAAC CTAGGGCTTC GGGTCCAGCA GAAGGGCACC TCAGAAACAG  
240

5 ACACCATCTG CACCTGTGAA GAAGGCTGGC ACTGTACGAG TGAGGCCTGT GAGAGCTGTG  
300

10 TCCTGCACCG CTCATGCTCG CCCGGCTTG GGGTCAAGCA GATTGCTACA GGGGTTCTG  
360

15 ATACCATCTG CGAGCCCTGC CCAGTCGGCT TCTTCTCAA TGTGTACATCT GCTTCGAAA  
420

20 AATGTCACCC TTGGACAAGC TGTGAGACCA AAGACCTGGT TGTGCAACAG GCAGGCACAA  
480

25 ACAAGACTGA TGTTGTCTGT GGTCCCCAGG ATCGGCTGA  
519

## 20 (2) INFORMATION FOR SEQ ID NO:5:

25 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 26 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: cDNA

35 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:  
(A) ORGANISM: PCR PRIMER

40 (vii) IMMEDIATE SOURCE:  
(B) CLONE: CD40 5' PRIMER

## 40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCGTCGACCA CCATGGTTCG TCTGCC  
26

## 45 (2) INFORMATION FOR SEQ ID NO:6:

50 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 28 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

55 (ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

60 (vi) ORIGINAL SOURCE:  
(A) ORGANISM: PCR PRIMER

IP

6

3

6

3

49

(vii) IMMEDIATE SOURCE:  
(B) CLONE: CD40 '3' PRIMER

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCGTCGACGT CTAGAGCCGA TCCTGGGG

28

10

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 40 base pairs  
15 (B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:  
25 (A) ORGANISM: PCR PRIMER

(vii) IMMEDIATE SOURCE:  
(B) CLONE: CD40 '3' DOWNSTREAM PRIMER

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ACAAGATCTG GGCTCTACGT ACTCAGCCGA TCCTGGGGAC

40

35 (2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:  
40 (A) LENGTH: 5 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:  
50 (A) ORGANISM: PENTAPEPTIDE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

55 Tyr Val Gly Pro Arg  
1 5

(2) INFORMATION FOR SEQ ID NO:9:

60 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 43 base pairs  
(B) TYPE: nucleic acid

50

(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

10 (A) ORGANISM: PCR PRIMER

(vii) IMMEDIATE SOURCE:

(B) CLONE: HUMAN IGG1/FC 5' PRIMER

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TATTAATCAT TCAGTAGGGC CCAGATCTTG TGACAAA CAC  
43

20 (2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 38 base pairs  
(B) TYPE: nucleic acid  
25 (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

30 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

35 (A) ORGANISM: PCR PRIMER

(vii) IMMEDIATE SOURCE:

(B) CLONE: HUMAN IGG1/FC 3' DOWNSTREAM PRIMER

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GCCAGCTTAA CTAGTT CATT TACCCGGAGA CAGGGAGA  
38

45 (2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

50 (A) LENGTH: 840 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

55 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

60 (vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

P

6

6

6

3

51

(vii) IMMEDIATE SOURCE:  
 (B) CLONE: CD40-L

5 (ix) FEATURE:

(A) NAME/KEY: CDS  
 (B) LOCATION: 46..831

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TGCCACCTTC TCTGCCAGAA GATACCATT CAACTTAAC ACAGC ATG ATC GAA  
 54

Met Ile Glu  
 1

ACA TAC AAC CAA ACT TCT CCC CGA TCT GCG GCC ACT GGA CTG CCC ATC  
 102

20 Thr Tyr Asn Gln Thr Ser Pro Arg Ser Ala Ala Thr Gly Leu Pro Ile  
 5 10 15

AGC ATG AAA ATT TTT ATG TAT TTA CTT ACT GTT TTT CTT ATC ACC CAG  
 150

25 Ser Met Lys Ile Phe Met Tyr Leu Leu Thr Val Phe Leu Ile Thr Gln  
 20 25 30 35

ATG ATT GGG TCA GCA CTT TTT GCT GTG TAT CTT CAT AGA AGG TTG GAC  
 198

30 Met Ile Gly Ser Ala Leu Phe Ala Val Tyr Leu His Arg Arg Leu Asp  
 40 45 50

AAG ATA GAA GAT GAA AGG AAT CTT CAT GAA GAT TTT GTA TTC ATG AAA  
 246

35 Lys Ile Glu Asp Glu Arg Asn Leu His Glu Asp Phe Val Phe Met Lys  
 55 60 65

ACG ATA CAG AGA TGC AAC ACA GGA GAA AGA TCC TTA TCC TTA CTG AAC  
 294

40 Thr Ile Gln Arg Cys Asn Thr Gly Glu Arg Ser Leu Ser Leu Leu Asn  
 70 75 80

TGT GAG GAG ATT AAA AGC CAG TTT GAA GGC TTT GTG AAG GAT ATA ATG  
 342

45 Cys Glu Glu Ile Lys Ser Gln Phe Glu Gly Phe Val Lys Asp Ile Met  
 85 90 95

TTA AAC AAA GAG GAG ACG AAG AAA GAA AAC AGC TTT GAA ATG CAA AAA  
 390

50 Leu Asn Lys Glu Glu Thr Lys Lys Glu Asn Ser Phe Glu Met Gln Lys  
 100 105 110 115

GGT GAT CAG AAT CCT CAA ATT GCG GCA CAT GTC ATA AGT GAG GCC AGC  
 438

55 Gly Asp Gln Asn Pro Gln Ile Ala Ala His Val Ile Ser Glu Ala Ser  
 120 125 130

AGT AAA ACA ACA TCT GTG TTA CAG TGG GCT GAA AAA GGA TAC TAC ACC  
 486

60 Ser Lys Thr Thr Ser Val Leu Gln Trp Ala Glu Lys Gly Tyr Tyr Thr  
 135 140 145

52

ATG AGC AAC AAC TTG GTA ACC CTG GAA AAT GGG AAA CAG CTG ACC GTT  
 534  
 Met Ser Asn Asn Leu Val Thr Leu Glu Asn Gly Lys Gln Leu Thr Val  
 150 155 160  
 5  
 AAA AGA CAA GGA CTC TAT TAT ATC TAT GCC CAA GTC ACC TTC TGT TCC  
 582  
 Lys Arg Gln Gly Leu Tyr Tyr Ile Tyr Ala Gln Val Thr Phe Cys Ser  
 165 170 175  
 10 AAT CGG GAA GCT TCG AGT CAA GCT CCA TTT ATA GCC AGC CTC TGC CTA  
 630  
 Asn Arg Glu Ala Ser Ser Gln Ala Pro Phe Ile Ala Ser Leu Cys Leu  
 180 185 190 195  
 15 AAG TCC CCC GGT AGA TTC GAG AGA ATC TTA CTC AGA GCT GCA AAT ACC  
 678  
 Lys Ser Pro Gly Arg Phe Glu Arg Ile Leu Leu Arg Ala Ala Asn Thr  
 200 205 210  
 20 CAC AGT TCC GCC AAA CCT TGC GGG CAA CAA TCC ATT CAC TTG GGA GGA  
 726  
 His Ser Ser Ala Lys Pro Cys Gly Gln Gln Ser Ile His Leu Gly Gly  
 215 220 225  
 25 GTA TTT GAA TTG CAA CCA GGT GCT TCG GTG TTT GTC AAT GTG ACT GAT  
 774  
 Val Phe Glu Leu Gln Pro Gly Ala Ser Val Phe Val Asn Val Thr Asp  
 230 235 240  
 30 CCA AGC CAA GTG AGC CAT GGC ACT GGC TTC ACG TCC TTT GGC TTA CTC  
 822  
 Pro Ser Gln Val Ser His Gly Thr Gly Phe Thr Ser Phe Gly Leu Leu  
 245 250 255  
 35 AAA CTC TGAACAGTGT CA  
 840  
 Lys Leu  
 260  
 40  
 (2) INFORMATION FOR SEQ ID NO:12:  
 45 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 261 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear  
 50 (ii) MOLECULE TYPE: protein  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:  
 Met Ile Glu Thr Tyr Asn Gln Thr Ser Pro Arg Ser Ala Ala Thr Gly  
 1 5 10 15  
 55 Leu Pro Ile Ser Met Lys Ile Phe Met Tyr Leu Leu Thr Val Phe Leu  
 20 25 30  
 60 Ile Thr Gln Met Ile Gly Ser Ala Leu Phe Ala Val Tyr Leu His Arg  
 35 40 45

53

Arg Leu Asp Lys Ile Glu Asp Glu Arg Asn Leu His Glu Asp Phe Val  
 50 55 60

5 Phe Met Lys Thr Ile Gln Arg Cys Asn Thr Gly Glu Arg Ser Leu Ser  
 65 70 75 80

Leu Leu Asn Cys Glu Glu Ile Lys Ser Gln Phe Glu Gly Phe Val Lys  
 85 90 95

10 Asp Ile Met Leu Asn Lys Glu Glu Thr Lys Lys Glu Asn Ser Phe Glu  
 100 105 110

Met Gln Lys Gly Asp Gln Asn Pro Gln Ile Ala Ala His Val Ile Ser  
 115 120 125

15 Glu Ala Ser Ser Lys Thr Thr Ser Val Leu Gln Trp Ala Glu Lys Gly  
 130 135 140

20 Tyr Tyr Thr Met Ser Asn Asn Leu Val Thr Leu Glu Asn Gly Lys Gln  
 145 150 155 160

Leu Thr Val Lys Arg Gln Gly Leu Tyr Tyr Ile Tyr Ala Gln Val Thr  
 165 170 175

25 Phe Cys Ser Asn Arg Glu Ala Ser Ser Gln Ala Pro Phe Ile Ala Ser  
 180 185 190

Leu Cys Leu Lys Ser Pro Gly Arg Phe Glu Arg Ile Leu Leu Arg Ala  
 195 200 205

30 Ala Asn Thr His Ser Ser Ala Lys Pro Cys Gly Gln Gln Ser Ile His  
 210 215 220

35 Leu Gly Gly Val Phe Glu Leu Gln Pro Gly Ala Ser Val Phe Val Asn  
 225 230 235 240

Val Thr Asp Pro Ser Gln Val Ser His Gly Thr Gly Phe Thr Ser Phe  
 245 250 255

40 Gly Leu Leu Lys Leu  
 260

45 (2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 73 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA to mRNA

55 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

60 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

IP

6

6

6

3

54

TGGTGGCGGA GGGTCAGGCG GAGGTGGTC CGGAGGCGGG GGTTCAAGTT CTGACAAGAT  
60

5 AGAAGATGAA AGG  
73

## (2) INFORMATION FOR SEQ ID NO:14:

10 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

20 (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

25 GGCCGCTCAG AGTTTGAGTA A  
21

## (2) INFORMATION FOR SEQ ID NO:15:

30 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 1425 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

40 (iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

45 (B) CLONE: Human CD40-L/FC2 (soluble CD40-L)

(ix) FEATURE:

(A) NAME/KEY: CDS  
(B) LOCATION: 4..1422

50 (ix) FEATURE:

(A) NAME/KEY: mat\_peptide  
(B) LOCATION: 79..1422

55 (ix) FEATURE:

(A) NAME/KEY: sig\_peptide  
(B) LOCATION: 4..78

60 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

IP

6

6

6

3

TAT ATG TTC CAT GTT TCT TTT AGA TAT ATC TTT GGA ATT CCT CCA CTG  
 48  
 Met Phe His Val Ser Phe Arg Tyr Ile Phe Gly Ile Pro Pro Leu  
 -25 -20 -15  
 5  
 ATC CTT GTT CTG CTG CCT GTC ACT AGC TCT GAC TAC AAA GAT GAC GAT  
 96  
 Ile Leu Val Leu Leu Pro Val Thr Ser Ser Asp Tyr Lys Asp Asp Asp  
 -10 -5 1 5  
 10  
 GAT AAA AGA TCT TGT GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA GCA  
 144  
 Asp Lys Arg Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala  
 10 15 20  
 15  
 CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC TTC CCC CCA AAA CCC  
 192  
 Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro  
 25 30 35  
 20  
 AAG GAC ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA TGC GTG GTG  
 240  
 Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val  
 40 45 50  
 25  
 GTG GAC GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG  
 288  
 Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val  
 55 60 65 70  
 30  
 GAC GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG  
 336  
 Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln  
 75 80 85  
 35  
 TAC AAC AGC ACG TAC CCG GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG  
 384  
 Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln  
 90 95 100  
 40  
 GAC TGG CTG AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC  
 432  
 Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala  
 105 110 115  
 45  
 CTC CCA GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC  
 480  
 Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro  
 120 125 130  
 50  
 CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC  
 528  
 Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr  
 135 140 145 150  
 55  
 AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC  
 576  
 Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser  
 155 160 165  
 60

56

GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC  
 624  
 Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr  
 170 175 180

5

AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC  
 672  
 Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr  
 185 190 195

10

AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC  
 720  
 Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe  
 200 205 210

15

TCA TGC TCC GTG ATG CAT GGT GGC GGA GGG TCA GGC GGA GGT GGG TCC  
 768  
 Ser Cys Ser Val Met His Gly Gly Gly Ser Gly Gly Gly Ser  
 215 220 225 230

20

GGA GGC GGG GGT TCA AGT TCT GAC AAG ATA GAA GAT GAA AGG AAT CTT  
 816  
 Gly Gly Gly Ser Ser Ser Asp Lys Ile Glu Asp Glu Arg Asn Leu  
 235 240 245

25

CAT GAA GAT TTT GTA TTC ATG AAA ACG ATA CAG AGA TGC AAC ACA GGA  
 864  
 His Glu Asp Phe Val Phe Met Lys Thr Ile Gln Arg Cys Asn Thr Gly  
 250 255 260

30

GAA AGA TCC TTA TCC TTA CTG AAC TGT GAG GAG ATT AAA AGC CAG TTT  
 912  
 Glu Arg Ser Leu Ser Leu Leu Asn Cys Glu Glu Ile Lys Ser Gln Phe  
 265 270 275

35

GAA GGC TTT GTG AAG GAT ATA ATG TTA AAC AAA GAG GAG ACG AAG AAA  
 960  
 Glu Gly Phe Val Lys Asp Ile Met Leu Asn Lys Glu Glu Thr Lys Lys  
 280 285 290

40

GAA AAC AGC TTT GAA ATG CAA AAA GGT GAT CAG AAT CCT CAA ATT GCG  
 1008  
 Glu Asn Ser Phe Glu Met Gln Lys Gly Asp Gln Asn Pro Gln Ile Ala  
 295 300 305 310

45

GCA CAT GTC ATA AGT GAG GCC AGC AGT AAA ACA ACA TCT GTG TTA CAG  
 1056  
 Ala His Val Ile Ser Glu Ala Ser Ser Lys Thr Thr Ser Val Leu Gln  
 315 320 325

50

TGG GCT GAA AAA GGA TAC TAC ACC ATG AGC AAC AAC TTG GTA ACC CTG  
 1104  
 Trp Ala Glu Lys Gly Tyr Tyr Met Ser Asn Asn Leu Val Thr Leu  
 330 335 340

55

GAA AAT GGG AAA CAG CTG ACC GTT AAA AGA CAA GGA CTC TAT TAT ATC  
 1152  
 Glu Asn Gly Lys Gln Leu Thr Val Lys Arg Gln Gly Leu Tyr Tyr Ile  
 345 350 355

60

IP

6

3

57

TAT GCC CAA GTC ACC TTC TGT TCC AAT CGG GAA GCT TCG AGT CAA GCT  
 1200

Tyr Ala Gln Val Thr Phe Cys Ser Asn Arg Glu Ala Ser Ser Gln Ala  
 360 365 370

5

CCA TTT ATA GCC AGC CTC TGC CTA AAG TCC CCC GGT AGA TTC GAG AGA  
 1248

Pro Phe Ile Ala Ser Leu Cys Leu Lys Ser Pro Gly Arg Phe Glu Arg  
 375 380 385 390

10

ATC TTA CTC AGA GCT GCA AAT ACC CAC AGT TCC GCC AAA CCT TGC GGG  
 1296

Ile Leu Leu Arg Ala Ala Asn Thr His Ser Ser Ala Lys Pro Cys Gly  
 395 400 405

15

CAA CAA TCC ATT CAC TTG GGA GGA GTA TTT GAA TTG CAA CCA GGT GCT  
 1344

Gln Gln Ser Ile His Leu Gly Gly Val Phe Glu Leu Gln Pro Gly Ala  
 410 415 420

20

TCG GTG TTT GTC AAT GTG ACT GAT CCA AGC CAA GTG AGC CAT GGC ACT  
 1392

Ser Val Phe Val Asn Val Thr Asp Pro Ser Gln Val Ser His Gly Thr  
 425 430 435

25

GGC TTC ACG TCC TTT GGC TTA CTC AAA CTC TGA  
 1425

Gly Phe Thr Ser Phe Gly Leu Leu Lys Leu  
 440 445

30

(2) INFORMATION FOR SEQ ID NO:16:

35 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 473 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Met Phe His Val Ser Phe Arg Tyr Ile Phe Gly Ile Pro Pro Leu Ile  
 -25 -20 -15 -10

45 Leu Val Leu Leu Pro Val Thr Ser Ser Asp Tyr Lys Asp Asp Asp Asp  
 -5 1 5

50 Lys Arg Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro  
 10 15 20

Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys  
 25 30 35

55 Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val  
 40 45 50 55

Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp  
 60 65 70

60 Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr

58

75

80

85

Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp  
 90 95 100

5

Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu  
 105 110 115

10

Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg  
 120 125 130 135

Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys  
 140 145 150

15

Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp  
 155 160 165

20

Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys  
 170 175 180

Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser  
 185 190 195

25

Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser  
 200 205 210 215

Cys Ser Val Met His Gly Gly Gly Ser Gly Gly Gly Ser Gly  
 220 225 230

30

Gly Gly Gly Ser Ser Ser Asp Lys Ile Glu Asp Glu Arg Asn Leu His  
 235 240 245

Glu Asp Phe Val Phe Met Lys Thr Ile Gln Arg Cys Asn Thr Gly Glu  
 250 255 260

35

Arg Ser Leu Ser Leu Leu Asn Cys Glu Glu Ile Lys Ser Gln Phe Glu  
 265 270 275

40

Gly Phe Val Lys Asp Ile Met Leu Asn Lys Glu Glu Thr Lys Lys Glu  
 280 285 290 295

Asn Ser Phe Glu Met Gln Lys Gly Asp Gln Asn Pro Gln Ile Ala Ala  
 300 305 310

45

His Val Ile Ser Glu Ala Ser Ser Lys Thr Thr Ser Val Leu Gln Trp  
 315 320 325

50

Ala Glu Lys Gly Tyr Tyr Thr Met Ser Asn Asn Leu Val Thr Leu Glu  
 330 335 340

55

Asn Gly Lys Gln Leu Thr Val Lys Arg Gln Gly Leu Tyr Tyr Ile Tyr  
 345 350 355

Ala Gln Val Thr Phe Cys Ser Asn Arg Glu Ala Ser Ser Gln Ala Pro  
 360 365 370 375

60

Phe Ile Ala Ser Leu Cys Leu Lys Ser Pro Gly Arg Phe Glu Arg Ile  
 380 385 390

Leu Leu Arg Ala Ala Asn Thr His Ser Ser Ala Lys Pro Cys Gly Gln  
 395 400 405

59

Gln Ser Ile His Leu Gly Gly Val Phe Glu Leu Gln Pro Gly Ala Ser  
410 415 420

5 Val Phe Val Asn Val Thr Asp Pro Ser Gln Val Ser His Gly Thr Gly  
425 430 435

Phe Thr Ser Phe Gly Leu Leu Lys Leu  
440 445

10 (2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

15 (A) LENGTH: 33 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

25 Arg Met Lys Gln Ile Glu Asp Lys Ile Glu Glu Ile Leu Ser Lys Ile  
1 5 10 15

Tyr His Ile Glu Asn Glu Ile Ala Arg Ile Lys Lys Leu Ile Gly Glu  
20 25 30

30 Arg

10

6

7

8

9

CLAIMS

What is claimed is:

5

1. An isolated DNA sequence encoding a CD40-L polypeptide that binds to CD40, wherein the DNA sequence is selected from the group consisting of:

(a) nucleotides 46 through 828, 184 through 828, or 193 through 762 of SEQ ID NO:11;

10 (b) DNA sequences which detectably hybridize to one of the sequences of (a) or their complementary strands under moderate stringency conditions; and

(c) DNA sequences which, due to degeneracy of the genetic code, encode a polypeptide encoded by any of the foregoing DNA sequences.

15

2. An isolated DNA sequence according to claim 1 encoding a soluble CD40-L polypeptide.

3. A recombinant expression vector comprising a DNA sequence according to claim 1.

20

4. A recombinant expression vector comprising a DNA sequence according to claim 2.

25

5. A host cell transformed or transfected with an expression vector according to claim 3.

6. A host cell transformed or transfected with an expression vector according to claim 4.

30

7. A process for preparing a CD40-L polypeptide, comprising culturing a host cell according to claim 5 under conditions promoting expression and recovering CD40-L polypeptide from the culture.

35

8. A process for preparing a CD40-L polypeptide, comprising culturing a host cell according to claim 6 under conditions promoting expression and recovering CD40-L polypeptide from the culture.

9. A purified biologically active CD40-L polypeptide composition comprising a sequence of amino acids encoded by a nucleotide sequence according to claim 1.

10. A purified biologically active CD40-L polypeptide composition according to claim 9, consisting essentially of human CD40-L.

11. A purified biologically active CD40-L polypeptide composition according to claim 10, selected from the group consisting:

- (a) the polypeptide defined by amino acids 1 through 261, inclusive, of the sequence set forth in SEQ ID NO:11;
- (b) the polypeptide defined by amino acids 47 through 261, inclusive, of the sequence set forth in SEQ ID NO:11; and
- (c) a polypeptide defined by a sequence beginning with an amino acid in the sequence between amino acid 47 and amino acid 51, inclusive, through and including an amino acid in the sequence between amino acid 239 and amino acid 261, inclusive, of the sequence set forth in SEQ ID NO:11.

12. A purified biologically active CD40-L polypeptide composition according to claim 11, wherein the CD40-L is a soluble CD40-L.

13. A soluble CD40-L according to claim 12, wherein the soluble CD40-L comprises amino acids 51 through 261 of SEQ ID NO:11.

14. A soluble CD40-L according to claim 13 wherein the CD40-L is an oligomer comprising two or more CD40-L extracellular regions.

15. A method of treating allergy, an allergic reaction, lupus, rheumatoid arthritis or graft versus host disease comprising administering an effective amount of a CD40 antagonist selected from the group consisting of a soluble CD40 protein, a CD40 fusion protein, a soluble monomeric CD40-L polypeptide, and combinations thereof.

16. A method of augmenting a vaccine response with an adjuvant, wherein the adjuvant is selected from the group consisting of a membrane-bound CD40-L polypeptide, oligomeric soluble CD40-L polypeptides, and combinations thereof.

17. A method for stimulating hybridoma cells to increase monoclonal antibody secretion, comprising administering an effective amount of a CD40 agonist selected

62

from the group consisting of CD40-L, membrane-bound CD40-L and oligomeric CD40-L.

18 An antibody immunoreactive with CD40-L or a CD40-L immunogen.

5

19. An antibody according to claim 16 which is a monoclonal antibody.

20. An antisense or sense oligonucleotide that can inhibit transcription or translation of CD40-L, comprising a sequence of at least about 12 nucleotides corresponding to a DNA sequence according to claim 1 or its DNA or RNA complement.

ip

□

□

6

3

1 CTTTCAGTCAGCATGATAGAAACATACAGCCAACCTCCCCAGATCCGTGGCAACTGGA 60  
 1 Met Ile Glu Thr Tyr Ser Gln Pro Ser Pro Arg Ser Val Ala Thr Gly 16

61 CTTCCAGCGAGCATGAAGATTTTATGTATTTACTTACTGTTTCTTATCACCCAAATG 120  
 17 Leu Pro Ala Ser Met Lys Ile Phe Met Tyr Leu Leu Thr Val Phe Leu Ile Thr Gln Met 36

121 ATTGGATCTGTGCTTTGCTGTATCTCATAGAACAGATTGGATAAGGTCGAAGAGGAA 180  
 37 Ile Gly Ser Val Leu Phe Ala Val Tyr Leu His Arg Arg Leu Asp Lys Val Glu Glu 56

181 GTAAACCTTCATGAAGATTTGTATTCTATAAAAAGCTAAAGAGATGCAACAAAGGAGAA 240  
 57 Val Asn Leu His Glu Asp Phe Val Phe Ile Lys Leu Lys Arg Cys Asn Lys Gly Glu 75

241 GGATCTTATCCTGCTGAACTGTGAGGAGATGAGAAGGCAATTGAAAGACCTTGTCAAG 300  
 77 Gly Ser Leu Ser Leu Leu Asn Cys Glu Glu Met Arg Arg Gln Phe Glu Asp Leu Val Lys 95

301 GATATCACGTTAACAAAGAACAGAGAAAAACAGCTTGAAATGCAAGAGGTGAT 360  
 97 Asp Ile Thr Leu Asn Lys Glu Glu Lys Lys Glu Asn Ser Phe Glu Met Gln Arg Gly Asp 115

361 GAGGATCCTCAAATTGCAGCACACGTTGTAAGCGAACAGTAATGCAGCATCCGTT 420  
 117 Glu Asp Pro Gln Ile Ala Ala His Val Val Ser Glu Ala Asn Ser Asn Ala Ala Ser Val 135

421 CTACAGTGGGCCAAGAAAGGATATTACCATGAAAGCACTTGGTAATGCTTGAAT 460  
 137 Leu Gln Trp Ala Lys Lys Gly Tyr Tyr Thr Met Lys Ser Asn Leu Val Met Leu Glu Asn 155

481 GGGAAACAGCTGACGGTTAAAAGAGAACGGACTCTATTATGTCTACACTCAAGTCACCTTC 540  
 157 Gly Lys Gln Leu Thr Val Lys Arg Glu Gly Leu Tyr Tyr Val Tyr Thr Gln Val Thr Phe 175

541 TGCTCTAACCGGGAGCCTCGAGTCACGCCATTCTCGTCGGCTCTGGCTGAAGCCC 600  
 177 Cys Ser Asn Arg Glu Pro Ser Ser Gln Arg Pro Phe Ile Val Gly Leu Trp Leu Lys Pro 195

601 AGCATTGGATCTGAGAGAACCTTACTCAAGGCCAAATACCCACAGTCTCTCCAGCTT 660  
 197 Ser Ile Gly Ser Glu Arg Ile Leu Leu Lys Ala Ala Asn Thr His Ser Ser Gln Leu 215

661 TGCGAGCAGCAGTCTGTCACTTGGCGGAGTGTGTTGAATTACAAGCTGGTCTTGTG 720  
 217 Cys Glu Gln Gln Ser Val His Leu Gly Gly Val Phe Glu Leu Gln Ala Gly Ala Ser Val 235

721 TTTGTCAACGTGACTGAAGCAAGCCAAGTGATCCACAGAGTTGGCTCTCATCTTGGC 780  
 237 Phe Val Asn Val Thr Glu Ala Ser Gln Val Ile His Arg Val Gly Phe Ser Ser Phe Gly 255

781 TTACTCAAACCTGAAACAGTGCCTGCCTAGGCTGCAGCAGGGCTGATGCTGGCAGTCT 840  
 257 Leu Leu Lys Leu 265

841 CCCCTATACACCAAGTCAGTTAGGCCTCCCTGTGAACTCCCTATTATAACCCCTA 900

901 GGATCCTCCTCATGGAGAACTATTATTATGTACCCCCAAGGCACATAGAGCTGGPATAA 960

961 GAGATTACAGGGCAGGCCAAATCCCAAGGGACCCCTGCTCCCTAAGAACCTAACCTG 1020

1021 AACACAGCAACCCCACTGATTCAAGACAACCAGAAAAGACAAAGCCATAATACACAGATGAC 1080

1081 AGAGCTCTGATGAAACACAGATAACTAATGAGGCACAGTTGTGTTATGGGTGTGT 1140

1141 CGTTCAATGGACAGTGTACTTGACTTACCCAGGGAAAGATGCAGAACGGCAACTGTGAGCCT 1200

1201 CAGCTCACAACTGTGTTATGGTTGACCTGGGCTCCCTGCGGCCCTAGTAGG 1250

1 TGCCACCTTCTGCCAGAAGATAACCATTTAACACAGCATGATCGAAACATAC 60  
 1 MetIleGluThrTyr 5

61 AACCAAACTTCTCCCCGATCTGCGGCCACTGGACTGCCATCAGCATGAAAATTTATG 120  
 6 AsnGlnThrSerProArgSerAlaAlaThrGlyLeuProIleSerMetLysIlePheMet 25

121 TATTTACTTACTGTTTTCTTATCACCCAGATGATTGGCTCAGCACCTTGCTGTAT 160  
 26 TyrLeuLeuThrValPheLeuIleThrGlnMetIleGlySerAlaLeuPheAlaValTyr 45

181 CTTCATAGAAGGTTGGACAGAGATAGAAGATGAAAGGAATCTTCATGACATTTGTATTC 240  
 45 LeuHisArgArgLeuAspLysIleGluAspGluArgAsnLeuHisGluAspPheValPhe 65

241 ATGAAACCGATAACAGAGATGCAACACAGGGAGAAAGATCCTTATCCTTACTGAACTGTGAG 300  
 65 MetLysThrIleGlnArgCysAsnThrGlyGluArgSerLeuSerLeuAsnCysGlu 85

301 GAGATTAAAGCCAGTTGAAAGGCTTGTGAAAGGATATAATGTTAACAAAGAGGAGACG 360  
 85 GluIleLysSerGlnPheGluGlyPheValLysAspIleMetLeuAsnLysGluGluThr 105

361 ARGAAAGAAACAGCTTGAAATGCAAAAGGTGATCAGAAATCCTCAAAATTGGCCACAT 420  
 105 LysLysGluAsnSerPheGluMetGlnLysGlyAspGlnAsnProGlnIleAlaAlaHis 125

421 GTCATARGTGAGGCCAGCAGTAAACACATCTGTGTTACAGTGGGCTGAAAGGATAC 480  
 125 ValIleSerGluAlaSerSerLysThrSerValLeuGlnIleAspLysClyTyr 145

481 TACACCATGAGCAACAACTTGGTAACCCCTGGAAAATGGAAACAGCTGACCGTTAAAGA 540  
 145 TyrThrMetSerAsnAsnLeuValThrLeuGluAsnGlyLysGlnLeuThrValLysArg 165

541 CAAGGACTCTATTATATCTATGCCAAGTCACCTCTGTCACCGGAAAGCTCGAGT 600  
 165 GlnGlyLeuTyrTyrIleTyrAlaGlnValThrPheCysSerAsnArgGluAlaSerSer 185

601 CAAGCTCCATTAAAGCCAGCCCTCTGCCAAGTCACCTCTGTCACCGGAAAGCTCGAGT 660  
 185 GluAlaProPheIleAlaSerLeuCysLeuLysSerProGlyArgPheGluAlaSerSer 205

661 CTCAGAGCTGCAAAATACCCACAGTCCGCCAACCTGCGGGCAACATCCATTCACTTG 720  
 205 LeuArgAlaAlaAsnThrHisSerSerAlaLysProCysGlyGlnGlnSerIleHisLeu 225

721 CGAGGAGTATTGCAACCCAGGTGCTTCGGTGTTCATGTCACCTGATCCAAAGC 780  
 225 GlyGlyValPheGluLeuGlnProGlyAlaSerValPheValAsnValThrAspProSer 245

781 CAAGTGAGCCATGGCACTGGCTCACGTCTTGGCTTACTCAAACTCTGAAACAGTGTCA 840  
 245 GlnValSerHisGlyThrGlyPheThrSerPheGlyLeuLeuLysLeu 265

841 CCTTGAGGCTGTGGAGCTGACGCTGGGAGTCITCAAAACAGCACAGCGGTTAACGC 900

901 CCACCCCCCTGTTAACTGCCTATTTAAACCCCTAGGATCCTCCTTATGGAGAACTATTTAT 950

## FIG. 3

1 M I E T Y N Q T S P R S A A T G L P I S M K I F M Y L L T V F L I T Q M I G S A L F A V Y L H R R L 50  
 5 M I E T Y S Q P S P R S V A T G L P A S M K I F M Y L L T V F L I T Q M I G S V L F A V Y L H R R L 54  
 51 D K I E D E R N L H E D F V F M K T I Q R C N T G E R S L S L N C E E I K S Q F E G F V K D I M L 100  
 55 D K V E E E V N L H E D F V F I K K L K R C N K G E G S L S L L N C E E M R R Q F E D L V K D I T L 104  
 101 N K B E E T K K E N S P F E M Q K G D Q N P Q I A A H V I S E A S S K T T S V L Q W A E K G Y Y T M S N 150  
 105 N K B E . K K E N S F E M Q R G D E D P Q I A A H V V S E A N S N A A S V L Q W A K K G Y Y T M K S 153  
 151 N L V T L E N G K Q L T V K R Q G L Y Y I Y A Q V T F C S N R E A S S Q A P F I A S L C L K S P G R 200  
 154 N L V M L E N G K Q L T V K R E G L Y Y V Y T Q V T F C S N R E P S S Q R P F I V G L W L K P S I G 203  
 201 F E R I L L R A A N T H S S A K P C G Q Q S I H L G G V F E L Q P G A S V F V N V T D P S Q V S H G 250  
 204 S E R I L L K A A N T H S S S Q L C E Q Q S V H L G G V F E L Q A G A S V F V N V T E A S Q V I H R 253  
 251 T G F T S F G L L K L 261  
 254 V G F S S F G L L K L 264

FIG. 4A

10E5 E- + CV1's

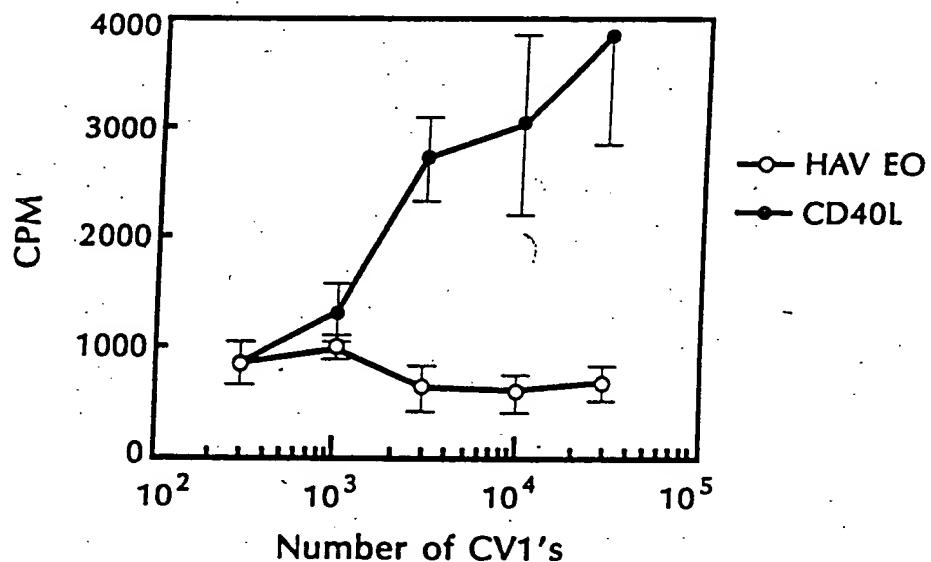


FIG. 4B

10E5 E- + IL-4 + CV1's

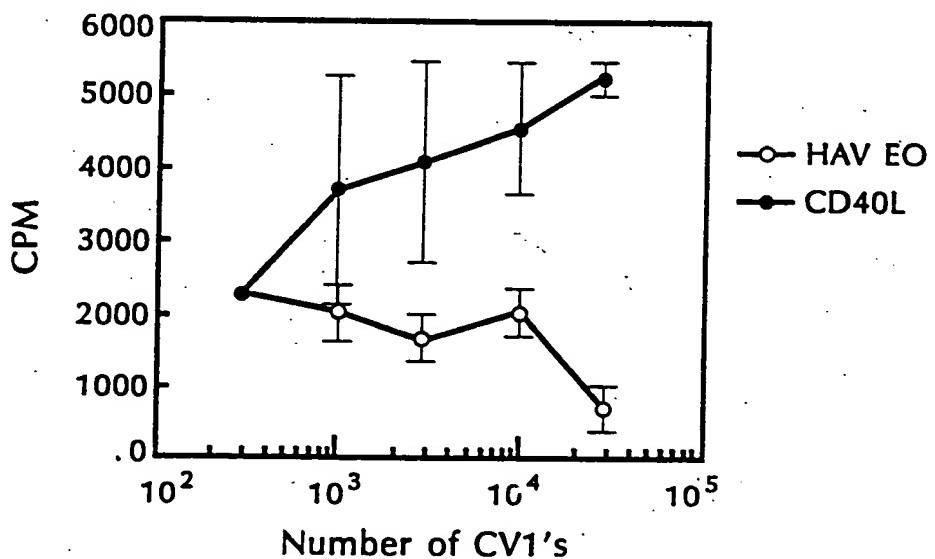
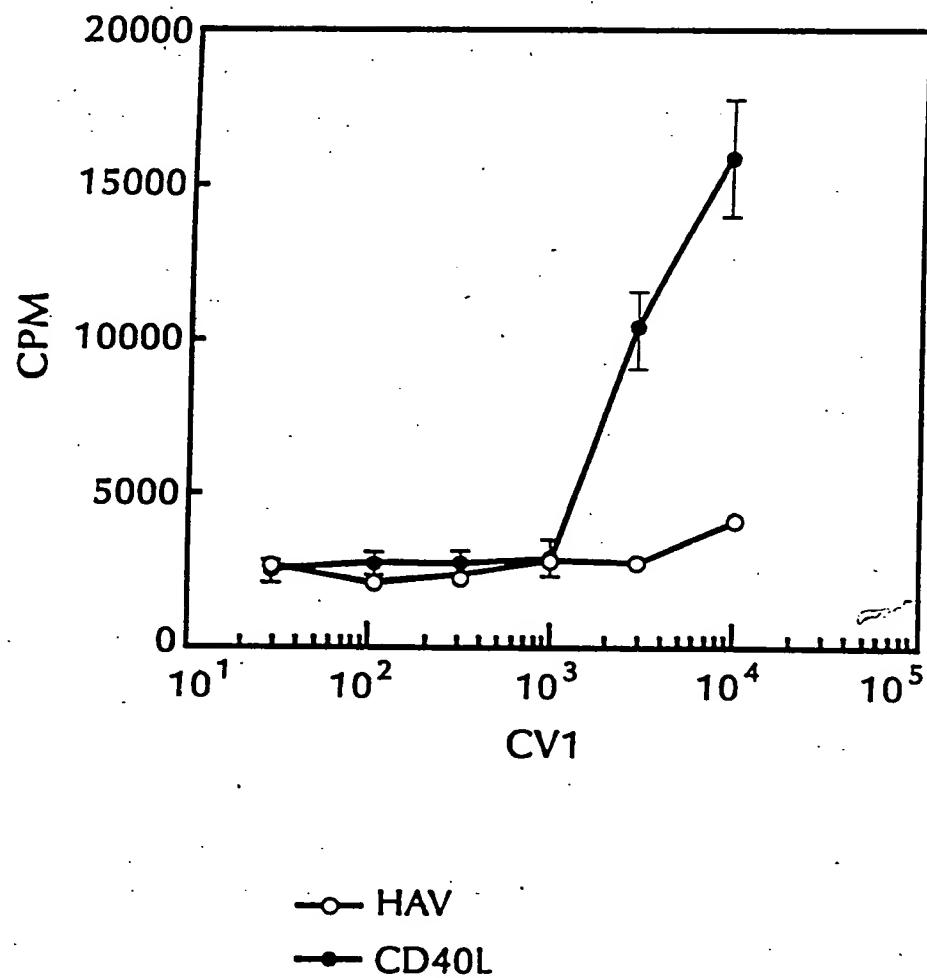


FIG. 5

IP

## PB E + IL-4 + CV1 d7 Proliferation

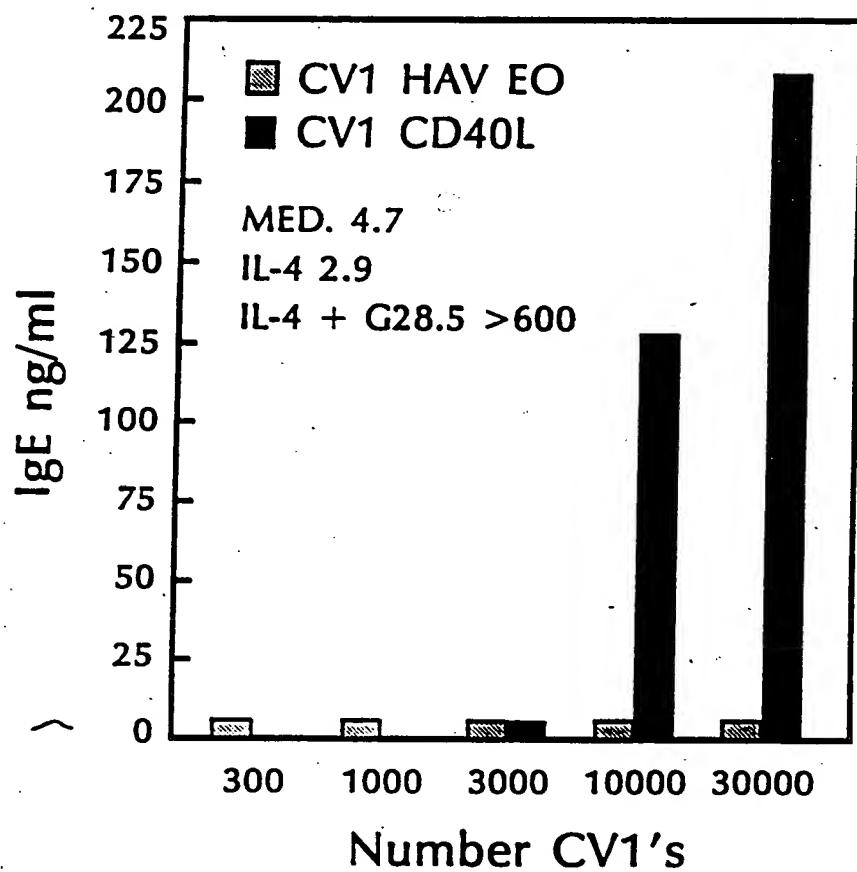


6

3

FIG. 6

S.CD23 in Day 6 Cultures S/N:  
10E5 E-/Well, IMDM + IL-4



**FIG. 7**

S.CD23 in Day 6 Cultures S/N:  
10E5 E-/Well, IMDM + IL-4

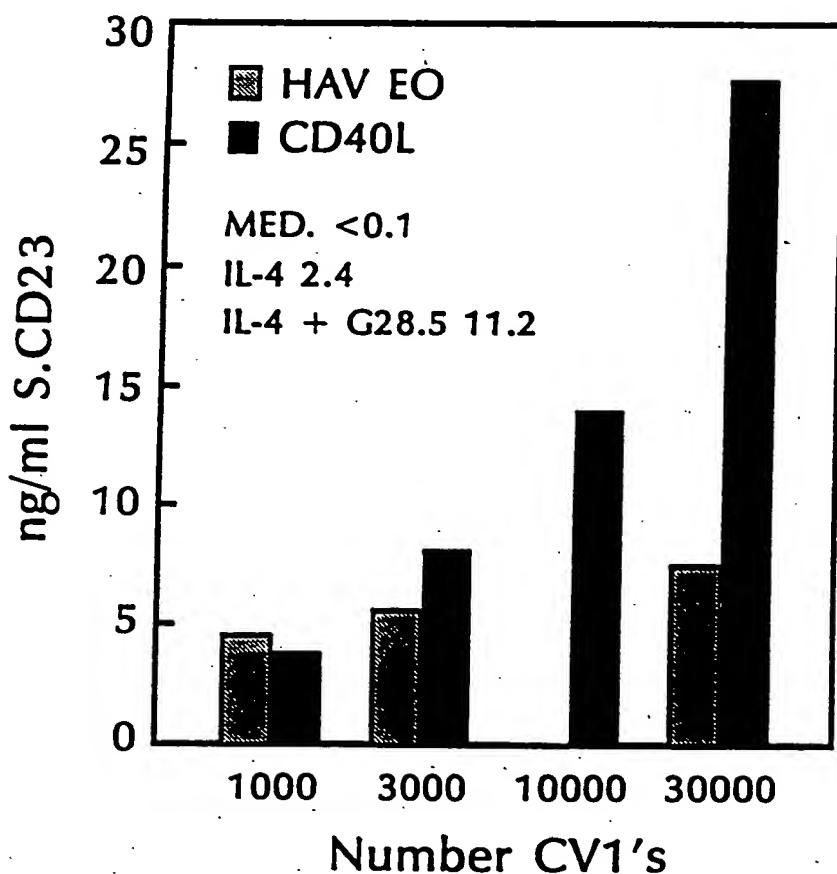


FIG. 8

Induction of B Cell Proliferation by  
CD40 Ligand Expressing CV-1 Cells (fixed)

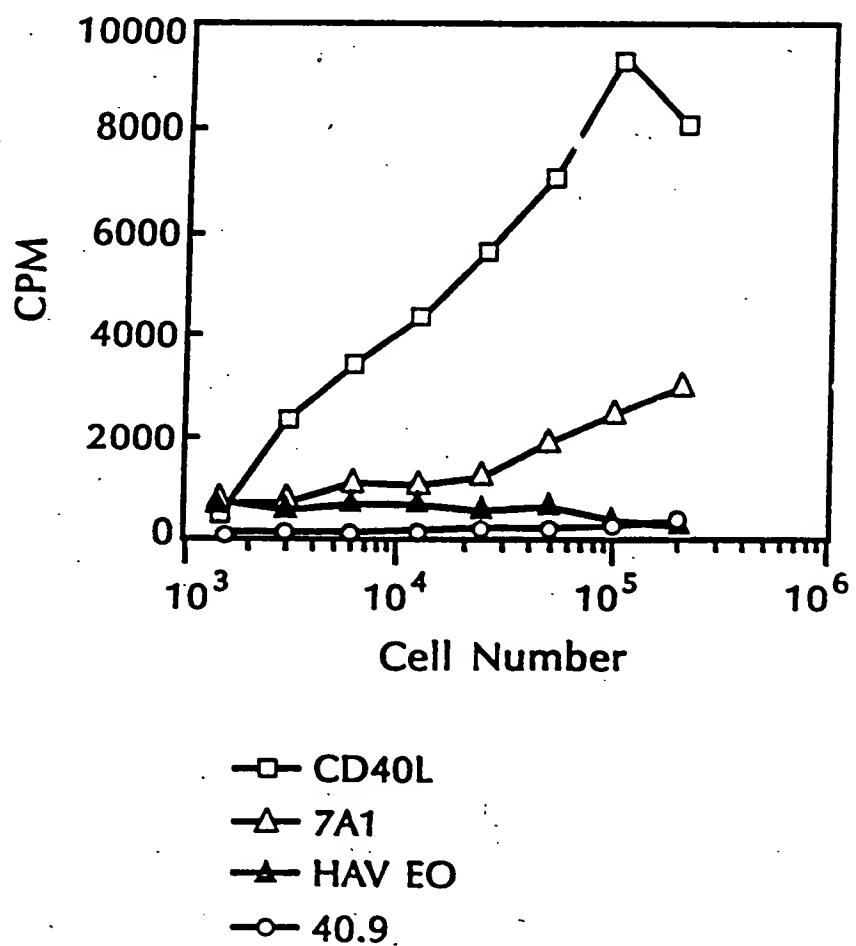
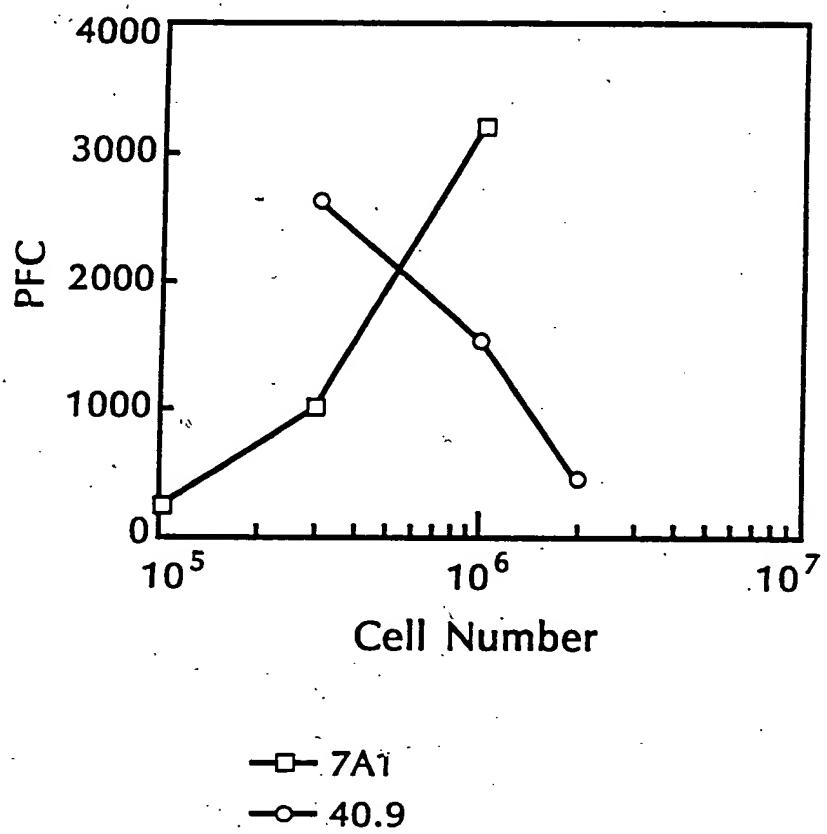
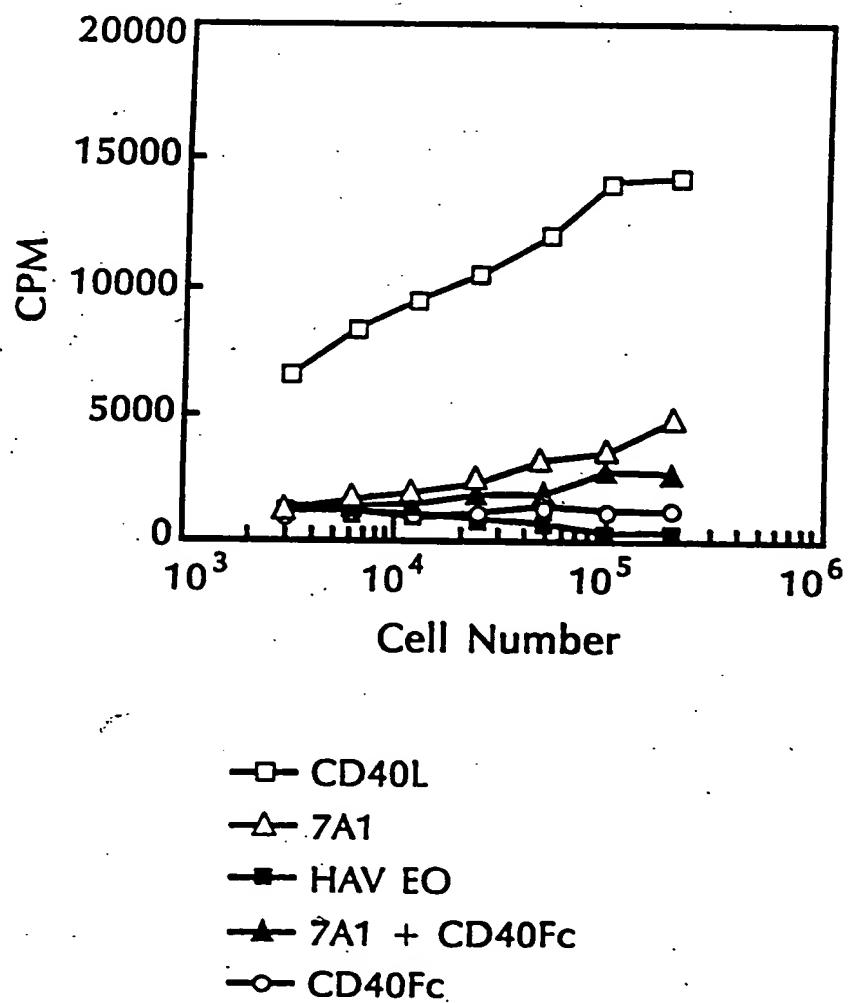
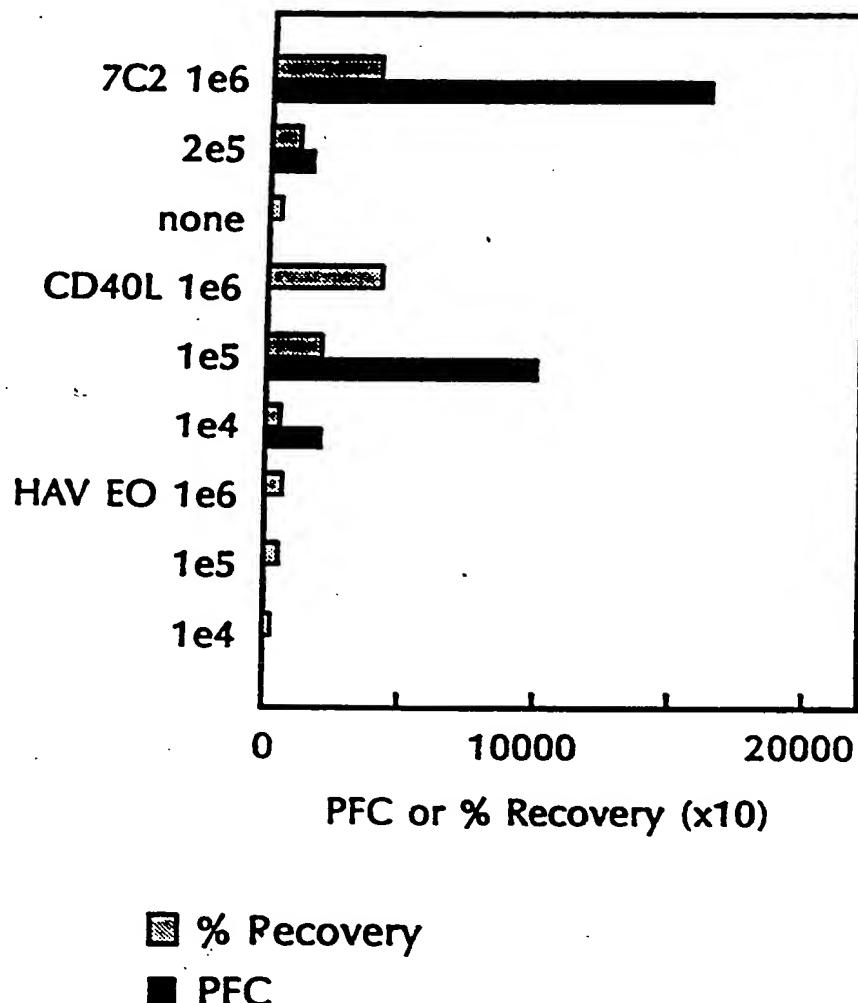


FIG. 9

Induction of Anti-SRBC PFC by EL4 40.9  
and 7A1 Th1 Cells (Fixed)



**FIG. 10****Induction of B Cell Proliferation by CD40 Ligand Expressing CV-1 Cells**

**FIG. 11****Induction of Anti-SRBC PFC by CD40 Ligand Expressing CV-1 Cells (fixed)**

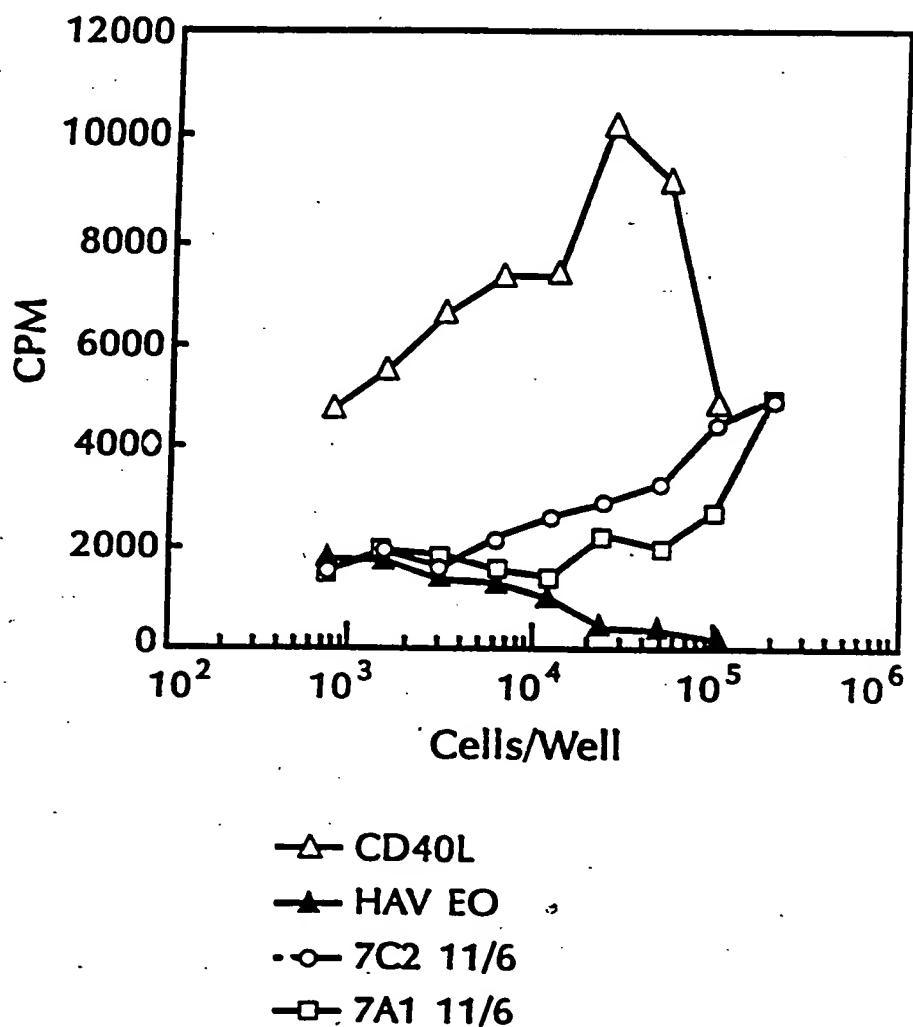
■ % Recovery  
■ PFC

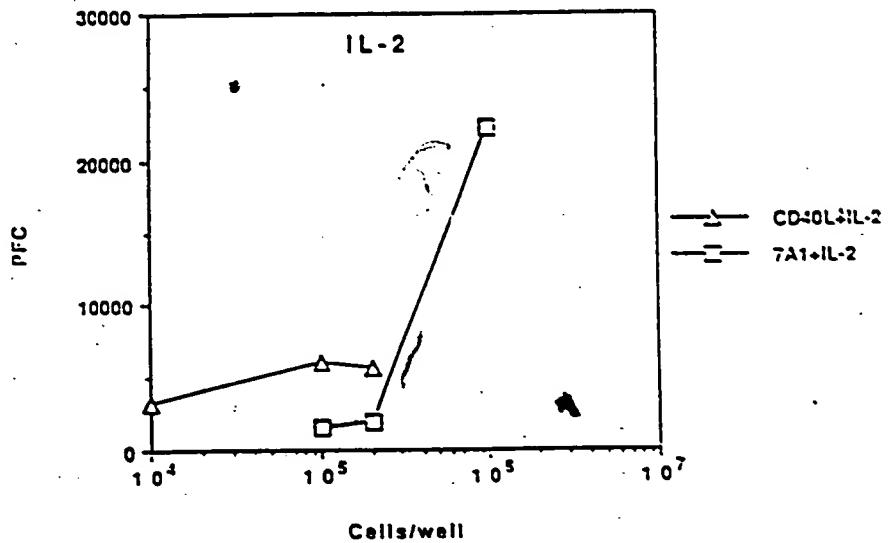
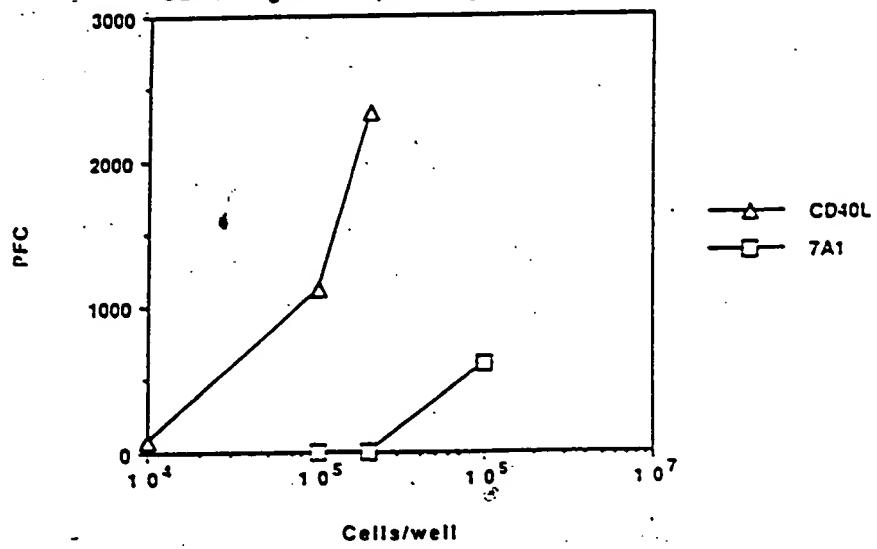
3194-11

12 / 14

FIG. 12

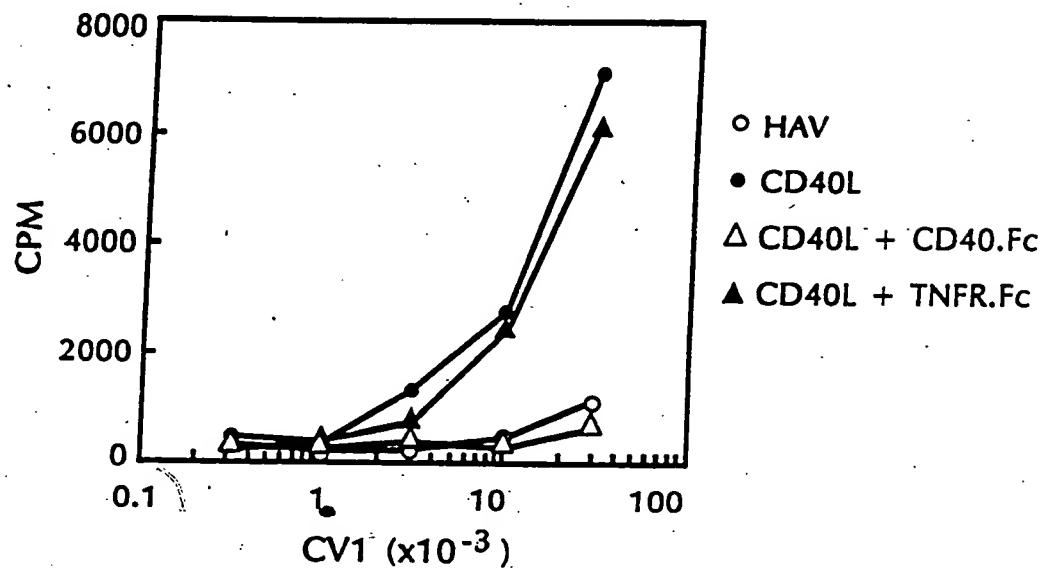
## Induction of Murine B Cell Proliferation by CD40 Ligand Expressing CV-1 Cels (fixed)



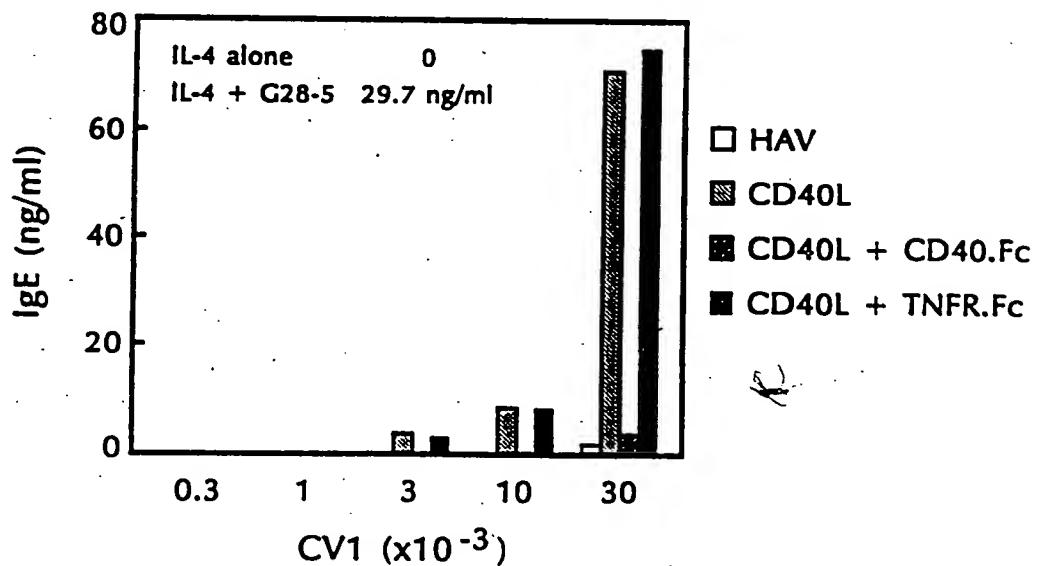
13/14  
*FIG. 13A*Induction of Antigen Specific PFC by  
CD40 Ligand Expressing CV-1 Cells (fixed)*FIG. 13B*

**FIG. 14A**

## Day 7 Proliferation of T-depleted PBM

**FIG. 14B**

## Day 10 IgE Secretion from T-depleted PBM



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US92/08990

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.  
US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/27; 530/388.22, 388.23, 389.2, 351; 435/70.3, 69.1, 69.5, 240.1, 252.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG  
search terms: CD40, ligand, antibodies, recombinant

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Nature, Volume 350, Issued 04 April 1991, M.L. Riordan et al., "Oligonucleotide-based therapeutics", pages 442-443, entire document.	20
Y	The EMBO Journal, Volume 8(5), issued 1989, I. Stamenkovic et al., "A B-lymphocyte activation molecule related to the nerve growth factor receptor and induced by cytokines in carcinomas", pages 1403-1410, entire document, especially page 1408, 2nd column, paragraphs 2-3.	1-14, 17-20
Y	Proceedings of the National Academy of Science, Vol. 84, issued December 1987, A. Aruffo and B. Seed, "Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system", pages 8573-8577, entire document, see especially page 8573, §2.	1-6

 Further documents are listed in the continuation of Box C.  See patent family annex.

Special categories of cited documents:	
"A"	document defining the general state of the art which is not considered to be part of particular relevance
"E"	earlier document published on or after the international filing date
"L"	document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O"	document referring to an oral disclosure, use, exhibition or other means
"P"	document published prior to the international filing date but later than the priority date claimed
"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"A"	document member of the same patent family

Date of the actual completion of the international search

13 January 1993

Date of mailing of the international search report

26 JAN 1993

Name and mailing address of the ISA/  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Authorized officer

LORRAINE M. SPECTOR, PH.D.

Facsimile No. NOT APPLICABLE

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US92/08990

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (5):

C07H 21/00; A61K 35/14; C07K 3/00, 7/00, 13/00; C12P 21/02, 21/06; C12N 15/00

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

536/27; 530/388.22, 388.23, 389.2, 351; 435/70.3, 69.1, 69.5, 240.1, 252.3